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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

One of the earliest events associated with the multistage pathogenesis of breast cancer is the activation of telomerase. Telomerase is a reverse transcriptase that elongates the ends of chromosomes and is required to prevent replicative senescence in proliferative cells. While not expressed in most adult human tissues, high levels of telomerase activity are present in most cancers. Ectopic expression of telomerase in many primary cell cultures is sufficient to bypass normal senescence. Cellular senescence can act as a terminal growth-control checkpoint, preventing the progression of pre-cancerous cells to malignancy. Blockade of telomerase activity in breast neoplasias should reintroduce this checkpoint, resulting in replication-dependent senescence of proliferating cells. Therefore, telomerase antagonists could potentially control growth and metastasis of residual cancer cells after surgery and chemotherapy. We are using two strategies to produce telomerase antagonists: 1) isolation of peptide aptamers that disrupt critical protein/protein interactions in the telomerase holoenzyme complex, and 2) development of an siRNA expression system to silence expression of hTERT and hTR RNAs.

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Introduction:

The telomerase holoenzyme is a multi-subunit complex consisting of a reverse transcriptase catalytic core (hTERT) and an RNA template component (hTR) together with other known and unknown accessory factors. We have identified critical protein/protein interactions in the telomerase holoenzyme required for catalytic activity. Here we are developing telomerase antagonists that target these interactions to functionally disrupt the holoenzyme. We have identified the molecular chaperones p23, and hsp90 as proteins that bind to the catalytic subunit of telomerase. Blockade of this interaction inhibits assembly of active telomerase in vitro. Also, a significant fraction of active telomerase from cell extracts is associated with p23 and hsp90. Consistent with in vitro results, inhibition of hsp90 function in cells blocks assembly of active telomerase. The association of human p23 with hTERT can be monitored in the yeast two hybrid system. This allows selective genetic screens to be developed for isolation of factors that specifically disrupt p23/hTERT complexes. We have shown that inhibition of hTERT/p23 association inhibits production of active telomerase. Therefore, we anticipate that small molecules that bind hTERT and prevent association with p23 will act as telomerase antagonists in cells. Our objective is to use selective screens in yeast to isolate such molecules.

It has very recently been demonstrated that small interfering RNA duplexes (siRNAs) can effectively and specifically mediate degradation of mRNAs in human cells. We have assessed the efficacy of siRNA mediated gene silencing in normal and tumor-derived human mammary epithelial cells. We find specific silencing of gene products can be achieved at high efficiency in these cells. Therefore, we are developing a system for delivery of siRNAs targeting hTERT and hTR.

Body:

Task 1. Isolate a pool of peptide aptamers that bind hTERT such that they block association with p23.

This task has been completed. We have isolated a panel of seven peptide aptamers that competitively associate with the p23-binding domain of hTERT.

Task 2. Identify those peptide aptamers isolated in task1 that will inhibit production of active telomerase in vitro.

This task is still in progress. The bottle-neck here has been optimization of an adequate in vitro system to test the aptamers. We found that co-synthesis of the aptamers with hTERT in rabbit reticulocyte lysates was problematic due to inhibition of hTERT synthesis. Separate synthesis of hTERT and the aptamers, followed by mixing, did not result in significant inhibition of hTERT activity. This is likely due to high levels of p23 in the rabbit reticulocyte lysates (RRLs) which associates with hTERT immediately upon synthesis. To allow the aptamers to directly compete for hTERT association during assembly of the holoenzyme, we are now taking advantage of the RRL-free in vitro assembly reaction we developed using purified recombinant chaperones (Holt et al.,

1999). For these assays, we will synthesize hTERT in RRL depleted of p23 (using p23 antibodies). Aptamers synthesized in separate in vitro transcription/translation reactions will be added to the in vitro assembly reactions to together with recombinant p23 over a range of relative molar ratios.

RNAi mediated inhibition of gene expression has been a very powerful tool for loss-of-function analysis in a number of model organisms. Recently, this technology has been successfully adapted for use in human cell culture by the observation that short RNA duplexes mimicking Dicer products are sufficient to induce RNAi without inducing a PKR/interferon response (Elbashir et al., 2001). In addition, technology has recently been developed allowing plasmid-based expression of effective siRNAs in human cell culture (Brummelkamp et al., 2002). Using siRNAs directed against endogenous proteins that are easily detectable by Western blot of whole cell lysates, we have demonstrated that siRNA is effective in human mammary epithelial cells (Fig. 1). We are currently designing siRNAs to target human hTERT and hTR. Our goal is to derive siRNAs expressed from adenoviral vectors that result in significant suppression of hTERT expression and hTR accumulation.

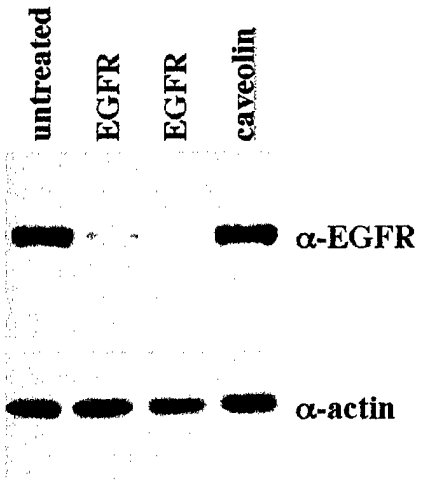

Figure 1. siRNA mediated inhibition of EGFR expression in human mammary epithelial cells. Synthetic siRNAs prepared according to Elbashir et al. were transfected into HME:hTERT cells using oligofectamine. 72 hours post transfection, cells were lysed and the indicated proteins were visualized by Western.

Key Research accomplishments:

-islolation of a panel of peptide aptamers that competitively association with the p23 binding domain of hTERT
-validation of siRNA technology in human mammary epithelial cells.

Reportable outcomes: none

Conclusions:

The reagents that are being developed will be useful in examining the biological consequences of inhibiting telomerase activity in human breast cancer cells. These reagents will have potential clinical applications to breast cancer therapy and prevention.

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Epigenetic Inactivation of RASSF1A in Lung and Breast Cancers and Malignant Phenotype Suppression

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Background: **The recently identified RASSF1 locus is located within a 120-kilobase region of chromosome 3p21.3 that frequently undergoes allele loss in lung and breast cancers. We explored the hypothesis that RASSF1 encodes a tumor suppressor gene for lung and breast cancers.** ***Methods:*** **We assessed expression of two RASSF1 gene products, RASSF1A and RASSF1C, and the methylation status of their respective promoters in 27 non-small-cell lung cancer (NSCLC) cell lines, in 107 resected NSCLCs, in 47 small-cell lung cancer (SCLC) cell lines, in 22 breast cancer cell lines, in 39 resected breast cancers, in 104 nonmalignant lung samples, and in three breast and lung epithelial cultures. We also transfected a lung cancer cell line that lacks RASSF1A expression with vectors containing RASSF1A complementary DNA to determine whether exogenous expression of RASSF1A would affect *in vitro* growth and *in vivo* tumorigenicity of this cell line. All statistical tests were two-sided.** ***Results:*** **RASSF1A messenger RNA was expressed in nonmalignant epithelial cultures but not in 100% of the SCLC, in 65% of the NSCLC, or in 60% of the breast cancer lines. By contrast, RASSF1C was expressed in all nonmalignant cell cultures and in nearly all cancer cell lines. RASSF1A promoter hypermethylation was detected in 100% of SCLC, in 63% of NSCLC, in 64% of breast cancer lines, in 30% of primary NSCLCs, and in 49% of primary breast tumors but in none of the nonmalignant lung tissues. RASSF1A promoter hypermethylation in resected NSCLCs was associated with impaired patient survival ($P = .046$). Exogenous expression of RASSF1A in a cell line lacking expression decreased *in vitro* colony formation and *in vivo* tumorigenicity.** ***Conclusion:*** **RASSF1A is a potential tumor suppressor gene that undergoes epigenetic inactivation in lung and breast cancers through hypermethylation of its promoter region. [J Natl Cancer Inst 2001;93:691–9]**

Allelic loss of human chromosome 3p is an early and frequent event in the development of several cancers, including lung and breast cancers *(1–5)*. Identification of a series of nested 3p21.3 homozygous deletions in small-cell lung cancers (SCLCs) directed an intensive effort to positionally clone tumor suppressor genes from a 630-kilobase (kb) region, which was recently narrowed to a 120-kb subregion by identification of a breast cancer homozygous deletion *(6–8)*. Sequencing the entire 630-kb region identified at least 25 genes, several of which may encode tumor suppressor genes for lung cancer *(7)*. Nine genes are located in or on the border of the breast cancer-defined subregion. One of these genes, which spans 7.6 kb of genomic DNA, has a predicted Ras-association domain and homology to the Ras-effector Norel (Fig. 1); it has, therefore, been termed "RASSF1" *(9,10)*.

The RASSF1 gene encodes two major transcripts, RASSF1A and RASSF1C, which are produced by alternative promoter selection and alternative messenger RNA (mRNA) splicing. RASSF1A is encoded by RASSF1 exons 1A, 1C, and 2–5. RASSF1C is encoded by RASSF1 exons 1–5 (Fig. 1). The start sites for RASSF1A and RASSF1C are approximately 2 kb apart and have two independent CpG island-containing putative promoter regions. RASSF1A is predicted to encode a 39-kd peptide that contains an N-terminal diacylglycerol (DAG)-binding domain and a Ras-association domain (Fig. 1). RASSF1C is predicted to encode a 32-kd peptide that lacks a DAG-binding domain but contains a Ras-association domain *(7,11)*. Immediately adjacent to the DAG-binding domain of RASSF1A is a sequence PxxP, which is the minimal sequence required for an src homology 3-binding domain. RASSF1A has a central linker that contains a number of prolines, as well as acidic and hydroxyl-bearing residues. These regions, called PEST sequences, are found in proteins that are rapidly turned over by ubiquitination-dependent pathways *(12)*. For RASSF1C, the amino-terminal region unique to this isoform is enriched for these PEST sequences. Within the PEST sequences common to both RASSF1A and RASSF1C is a serine residue that is phosphorylated *in vitro* by DNA-dependent ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia-related kinases *(13)*. The Ras-association domain is more than 50% identical and more than 70% similar to the carboxyl terminal 225 residues of mouse Norel *(10)*. The Ras-association domain, consisting of a core of 90 amino acids, is flanked on the amino terminal side by a region homologous with a region found in Norel and *Caenorhabditis elegans* orthologue T24F1.3 protein.

In this article, we characterized RASSF1A and RASSF1C as potential tumor suppressor genes in lung and breast cancers.

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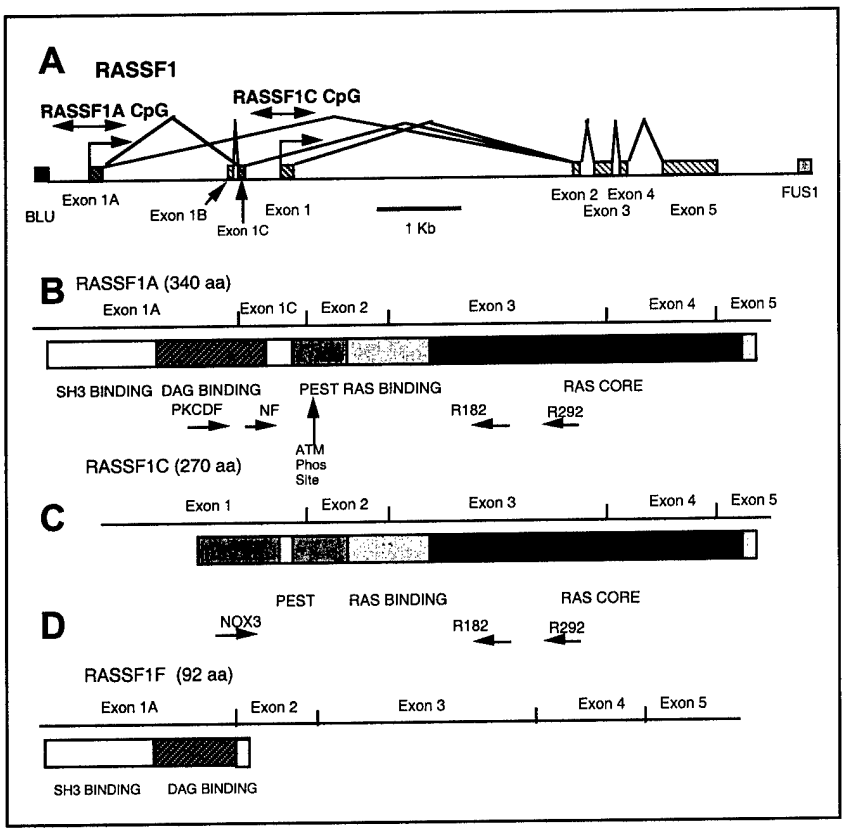

Fig. 1. Map of the RASSF1 locus, transcripts, and protein domains. **A)** The exon–intron structure of the RASSF1 locus with the location of the CpG islands in the predicted promoter regions (the locations of which are shown by **double-headed arrows**) of RASSF1A and RASSF1C. RASSF1A transcription is predicted to come from the most centromeric promoter region located within a CpG island and begins with exon 1A. RASSF1F also commences at this promoter but is missing exon 1C. Transcription of RASSF1C is predicted to begin in the most telomeric promoter region, which is approximately 2 kilobases from that of RASSF1A and begins with exon 1. **Blocks** represent exons; **lines** represent introns. **B)** Schematic of the RASSF1A transcript and predicted protein-sequence domains. The location of the various primers (PKCDF, NF, R182, and R292) used for isoform-specific reverse transcription (RT)–polymerase chain reaction (PCR) analyses are indicated. **Tick marks** identify the exon boundaries. The potential src homology 3 (SH3)-binding region, putative diacylglycerol (DAG)-binding domain, PEST sequence, Ras-association domain, and ataxia-telangiectasia-mutated (ATM) phosphorylation site are labeled. **C)** Schematic of the RASSF1C transcript and predicted protein-sequence domains. The locations of the various primers (NOX3, R182, and R292) used for isoform-specific RT–PCR analyses are indicated. **D)** Schematic of the RASSF1F transcript and predicted protein-sequence domains.

Because loss of gene expression can be caused by tumor-acquired aberrant methylation, we assessed the methylation status of the RASSF1A promoter region in these tumors *(14)*. In addition, we tested the ability of RASSF1A to suppress the malignant phenotype. Previously, Dammann et al. *(15)* showed that the RASSF1A promoter is hypermethylated in lung cancer cells and that exogenous expression of RASSF1A suppresses tumorigenesis in nude mice. We have confirmed and extended those findings by analyzing the expression and methylation status of both the RASSF1A and RASSF1C genes in lung and breast cancers.

Methods

Patient Population

Resected lung tumor samples and clinical data were collected from patients after obtaining appropriate institutional review board approval and patients' written informed consent. Primary tumor samples and corresponding noninvolved lung tissues were obtained from 107 patients with non-small-cell lung carcinoma (NSCLC) who had received curative resection surgery at the Prince Charles Hospital, Brisbane, Australia, from June 1990 through March 1993, and for whom clinical and survival data of 5 or more years were available *(16,17)*. Among the 107 patients, there were 76 males and 31 females (range, 28–81 years; mean age, 61 years at diagnosis). Among the patients, 61 had stage I cancers, 21 had stage II, 24 had stage IIIA, and one had stage IIIB *(18)*. Histologically, there were 45 adenocarcinomas, 43 squamous cell carcinomas, 11 adenosquamous carcinomas, four large-cell carcinomas, three atypical carcinoids, and one typical carcinoid. Ninety-eight patients were smokers, with a mean exposure of 31 pack-years, and nine were never smokers or nonsmokers.

We also obtained 39 primary breast tumors from patients aged 31–84 years undergoing breast cancer treatment in The University of Texas Southwestern Hospital system. Among the patients, three had stage I cancers, 15 had stage IIA, two had stage IIB, eight had stage IIIA, five had stage IIB, and six had stage IV. Histologically, there were 30 infiltrating ductal carcinomas, four invasive lobular carcinomas, one lobular carcinoma *in situ*, two ductal carcinomas *in situ*, and two breast adenocarcinomas at metastatic sites. Clinical information was obtained by retrospective review of clinical records.

Cell Lines and Cell Cultures

Lung and breast tumor cell lines generated by us have been described previously *(19–21)*. Complementary DNAs (cDNAs) and genomic DNAs were obtained from cell lines, most of which have been deposited in the American Type Culture Collection (ATTC) (Manassas, VA), that represented the spectrum of lung cancer histologies. These cell lines include the following: (all Hxxxx lines have the prefix National Cancer Institute [NCI]-) SCLCs (i.e., H69, H82, H128, H146, H182, H187, H196, H209, H249, H289, H290, H345, H378, H524, H526, H592, H735, H738, H740, H748, H774, H841, H847, H862, H889, H1092, H1105, H1184, H1304, H1339, H1450, H1607, H1618, H1672, H1688, H1963, H2028, H2029, H2081, H2108, H2171, H2195, H2227, and HCC970) and NSCLCs (i.e., H23, H28, H125, H157, H226, H358, H720, H727, H838, H920, H1155, H1299, H1437, H1466, H1573, H1648, H1770, H1792, H1819, H1838, H1993, H2009, H2052, H2077, H2087, H2347, H2452, H2882, H2887, HCC44, HCC78, HCC95, HCC193, HCC515, HCC827, and HCC1171). Breast cancer cell lines used in these studies were the following: HTB19, HTB20, HTB22, HTB23, HTB24, HTB25, HTB26, HTB27, HTB121, HTB130, HTB131, HTB132, HTB133 (all HTB lines are available from the ATCC), HCC38, HCC70, HCC202, HCC712, HCC1007, HCC1143, HCC1187, HCC1395, HCC1419, HCC1428, HCC1500, HCC1569, HCC1739, HCC1806, HCC1937, HCC1599, HCC1954, HCC2157, HCC2185, HCC2218, HCC2688, and HCC2713. Normal human bronchial epithelial (NHBE) and small-airway epithelial (SAE) cell cultures were obtained from Clonetics (San Diego, CA) and were grown and harvested as directed by the vendor.

Expression Analysis of RASSF1 Isoforms

The identification of the RASSF1 gene (initially called 123F2) and its major isoforms RASSF1C and RASSF1A was reported as part of the overall characterization of the genes in the larger 630-kb 3p21.3 homozygous-deletion region *(7)* (Fig. 1, A–C).

Sequence information from exons 1A and 3 was used to design the forward primer PKCDF (5′-GGCGTCGTGCGCAAAGGCC-3′) and the reverse primer R182 (5′-GGGTGGCTTCTTGCTGGAGGG-3′) (Fig. 1, C). This primer pair

was used in reverse transcription (RT)–polymerase chain reaction (PCR) screens of lung, heart, and pancreatic tissue-specific cDNA libraries (Clontech Laboratories, Inc., Palo Alto, CA). The conditions used TaqGold (The Perkin-Elmer Corp., Norwalk, CT) with 1× TaqGold buffer adjusted to 2 m*M* $MgCl_2$. All reactions used a 70 °C–60 °C touchdown, with 5% dimethyl sulfoxide for 35 rounds (denaturation for 30 seconds, annealling for 30 seconds, and extension for 60 seconds) of PCR. The RASSF1A cDNA sequence is identical to that of the RASSF1C cDNA from the second exon to the carboxyl terminus, but the two cDNAs have different 5′ exons (RASSF1A, GenBank Accession #AF102770: exons 1A and 1C; RASSF1C, GenBank Accession #AF040703: exon 1 [Fig. 1]). We also isolated tissue-specific isoforms from the heart (RASSF1D, GenBank Accession #AF102771) and the pancreas (RASSF1E, GenBank Assession #AF102772) cDNA libraries.

Primers derived from exon–intron junctions (sequences available online at the Journal website) were used for genomic DNA single-strand conformation polymorphism mutation analysis of the coding regions of RASSF1A on a panel of NSCLC, SCLC, and breast cancer cell line DNAs. DNA was prepared from tumors and cell lines by standard methods *(22)*, and aberrantly migrating fragments were sequenced as described previously *(16,23)*.

RNA Analysis

Isoform-specific RT–PCR assays were used for analysis of RASSF1A and RASSF1C expression. Primers for RASSF1C were Nox3 (5′-CTGCAGCCAAGAGGACTCGG-3′) and R182 and for RASSF1A were either PKCDF or NF (5′-TGCAAGTTCACCTGCCAC-3′) and R182 (Fig. 1, C). Total RNA was isolated from previously described lung and breast cancer cell lines grown in RPMI-1640 medium supplemented with 5% fetal bovine serum (complete medium) *(19–21)* by Trizol extraction (Life Technologies, Inc. [GIBCO BRL], Rockville, MD). Four micrograms of total RNA was reverse transcribed by use of GIBCO-BRL Superscript First Strand cDNA Kit. All cDNA preparations were tested for the ability to amplify a nontranscribed genomic sequence immediately upstream of the first exon of the RASSF1A transcript. Any cDNAs that produced a product from this sequence were discarded because they were contaminated with genomic DNA.

We also assessed the expression of RASSF1A after exposure to 5-aza-2′-deoxycytidine, a drug that inhibits DNA methylation. We exposed subconfluent cultures of the RASSF1A-nonexpressing NSCLC line NCI-H157 to 0.5 μ*M* 5-aza-2′-deoxycytidine for 48 hours, after which we isolated total RNA and performed RT–PCR for RASSF1A, RASSF1C, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RT–PCR of GAPDH transcripts was performed with the use of forward primer GAPDH-C (5′-CATGACAACTTTGGTATCGTG-3′) and reverse primer GAPDH-D (5′-GTGTCGCTGTTGAAGTCAGA-3′). RT–PCR products were separated by agarose gel electrophoresis and visualized after staining with ethidium bromide.

Methylation Analysis

The methylation status of the presumed RASSF1A and RASSF1C promoter regions was determined by methylation-specific PCR. Genomic DNAs from lung cancer cell lines not expressing RASSF1A (NCI lines H1299, H1184, H1304, H841, H2108, and H128) or expressing RASSF1A (H1792 and H2009) were modified by sodium bisulfite treatment as described previously *(24,25)*. Bisulfite treatment converts cytosine bases to uracil bases but has no effect on methylcytosine bases. PCR amplification followed by sequencing of the PCR fragments identifies specific CpG dinucleotides in the promoter region that are modified by methylation *(24,26,27)*. PCR primers (sequences available online at the Journal website) were designed to amplify genomic sequences in the presumed promoter regions of RASSF1A (cosmid Luca12; GenBank Accession #AC002481 nucleotides 17 730–18 370) and RASSF1C (GenBank Accession #AC002481 nucleotides 21 022–21 152 and 21 194–21 332). The resulting PCR fragments were sequenced by automated fluorescence-based DNA sequencing to determine the methylation status.

The data on CpG methylation in RASSF1A-nonexpressing lung cancer cell lines (data available online at the Journal website) were used to design methylation-specific PCR *(24)* primers for the RASSF1A 5′ promoter region: The primers to detect the methylated form were 5′-GGGTTTTGCGAGAGCGCG-3′ (forward) and 5′-GCTAACAAACGCGAACCG-3′ (reverse), and the primers to detect the unmethylated form were 5′-GGTTTTGTGAGAGTGTGTTTAG-3′ (forward) and 5′-CACTAACAAACACAAACCAAAC-3′ (reverse). Each primer set generated a 169-base-pair (bp) product. Methylation-specific PCR cycling conditions consisted of one incubation of 15 minutes at 95 °C, followed by 40 cycles of a 30-second denaturation at 94 °C, 50 seconds at an annealing temperature (64 °C for methylation-specific and 59 °C for unmethylated-specific primers), a 30-second extension at 72 °C, and a final extension at 72 °C for 10 minutes. PCR products were separated in 2% agarose gels. Lymphocyte DNA, methylated *in vitro* by CpG (*Sss*I) methylase (New England Biolabs, Inc., Beverly, MA) following the manufacturer's directions, was used as a positive control. A water blank was used as a negative control.

Generation of Transfectants

RASSF1A cDNA was cloned into pcDNA3.1+ (Invitrogen Corp., Carlsbad, CA), resequenced to confirm that the cDNAs were in the correct orientation and reading frame, transcribed, and translated *in vitro* with commercial kits (Clonetech Laboratories, Inc.). The expression vector containing RASSF1A produced a 42-kd protein, and the vector containing RASSF1C produced a 32-kd protein on sodium dodecyl sulfate–polyacrylamide gels, close to their respective predicted molecular masses of 39 and 32 kd (data not shown). Expression vectors in pcDNA3.1 for mutant and wild-type p53 and their transfection and activity have been described previously *(28)*.

The RASSF1A expression vector was transfected into NSCLC NCI-H1299 cells expressing RASSF1C, but not RASSF1A, by use of Lipofectamine plus (Life Technologies, Inc.) according to the manufacturer's recommendations. For transient transfection studies, approximately 5×10^5 NSCLC NCI-H1299 cells, harvested from 80%–90% confluent cultures in complete medium, were transfected with 1 μg of purified plasmid DNA. Samples were plated in a minimum of triplicate, and cells were collected 48 hours after transfection. Because the pcDNA3.1+ expression vector contains a neomycin resistance gene, clones expressing RASSF1A were selected in complete medium supplemented with G418 (800 μg/mL). Stable clones were maintained in complete medium supplemented with G418 (600 μg/mL). We confirmed that the clones were expressing the transfected RASSF1A gene by isolating total RNA from individual clones and performing RT–PCR as described above. We also transfected NCI-H1299 cells with the vector containing no inserts and isolated stable clones.

The RASSF1A and RASSF1C cDNAs were also cloned in the retroviral vector pBABEpuro and were resequenced to confirm that the genes were in the correct sequence and orientation *(29)*. Virus was prepared in the 293 cell-based Phoenix packaging cell line as described previously *(29)* from cells infected either with the vector alone or with constructs containing the RASSF1A or RASSF1C cDNA. Culture supernatants were collected by centrifugation at 500*g* at 37 °C for 10 minutes and used to infect NSCLC NCI-H1299 cells as described previously *(29)*. Because the viral vector contains a puromycin resistance gene, infected cells were selected with 1 μg/mL of puromycin for 7 days. Cells surviving the selection and containing the transgene were pooled, and total cell extracts were made. Western blot analysis was performed as described previously *(30)* to verify protein expression of the transfected genes. The protein bands were visualized with the Pierce SuperSignal Kit (Pierce Chemical Co., Rockford, IL).

Tumorigenicity Testing

The *in vitro* growth characteristics of NSCLC NCI-H1299 clones that express RASSF1A were tested for anchorage-dependent and anchorage-independent (soft agar) growth. After 48 hours of growth in nonselective medium, transiently transfected NSCLC NCI-H1299 cells were detached with trypsin and diluted, usually 10- to 25-fold, in complete medium containing 800 μg/mL of G418 and plated into fresh 100-mm dishes. The medium was changed twice weekly. After 14 days, the medium was removed, the plates were washed with phosphate-buffered saline (PBS), and the colonies were stained with 1% methylene blue in 50% (vol/vol) ethanol. For the anchorage-independent, soft agar-growth assays, 1000 RASSF1A-expressing cells were suspended and plated in 0.33% Noble agar (Sigma Chemical Co., St. Louis, MO) in complete medium supplemented with 600 μg/mL G418 and layered over a 0.50% agar base in complete medium. After 21 days, colonies greater than 0.2 mm in diameter were counted.

For retrovirally infected cells, anchorage-independent growth assays were performed as follows: 10 000 viable selected cells from each infection were plated in 0.33% soft agar over a 0.50% agar base in Dulbecco's modified Eagle medium (Life Technologies, Inc.) with 10% heat-inactivated fetal bovine serum. After 21 days, colonies greater than 0.2 mm in diameter were counted.

We also tested the ability of RASSF1A-infected cells to grow *in vivo* in nude mice. Male BALB/c nude (nu/nu) 3- to 6-week-old mice (Charles River Laboratories, Wilmington, DE) were irradiated on day 0 of the experiment in groups of five animals by a 5-minute exposure to 350 cGy from a cesium source. The

next day, each mouse was given an injection subcutaneously on its flank with 0.2 mL of sterile PBS containing 10^7 viable parental, vector control, or RASSF1A retroviral-infected NSCLC NCI-H1299 tumor cells. Mice were monitored every 2–3 days for tumor size; once tumors reached greater than 1500 mm^3, the mice were killed. All animal care was in accord with institutional guidelines.

Antibody Preparation

The entire RASSF1C open reading frame was used to make a glutathione *S*-transferase (GST) fusion protein, which was expressed in *Escherichia coli* by use of an established procedure *(31)*, and was used to make rabbit polyclonal antibodies to be described in detail elsewhere. For the western blot analysis, the antiserum was used at a 1 : 1000 dilution in 5% nonfat milk in PBS. Specificity was determined by western blotting of H1299 cells transfected with vector (negative control) and the various RASSF1-expression constructs (e.g., *see* Fig. 6).

Statistical Analysis

Statistical analysis was performed by use of χ^2 and Fisher's exact tests for differences between groups. Overall survival curves were calculated by use of the Kaplan–Meier method, and survival curves were compared with the log-rank statistic *(32)*. All analyses, including univariate, multivariate, and Cox analyses, were performed by use of SPSS Windows version 9.0.1 (SPSS Inc., Chicago, IL). All statistical tests were two-sided.

Results

Characterization of the RASSF1 Gene

To determine if the RASSF1A gene was mutated in lung and breast cancers, we performed extensive mutational analysis of the RASSF1A isoform with the use of single-strand conformation polymorphism assays on genomic DNA. We had previously found no RASSF1C mutations in 77 lung cancer cell line samples *(7)*. By use of the RASSF1A sequence as a reference, we found several polymorphisms, including the following: codon 21 (AAG to CAG), Lys to Gln; codon 28 (CGT to CGA), no amino acid change; codon 49 (GGC to GGT), no amino acid change; codon 53 (CGC to TGC), Arg to Cys; codon 129 (GAC to GAG), Asp to Glu; codon 133 (GCT to TCT), Ala to Ser; and codon 325 (TAT to TGT), Tyr to Cys.

Expression of RASSF1A and RASSF1C in Lung and Breast Cancer Cell Lines

RASSF1 is located within a region frequently affected by allele loss during growth of lung, breast, head and neck, kidney, and cervical tumors *(1–5)*. We investigated whether RASSF1A and RASSF1C are expressed in lung and breast cancer cell lines. We used isoform-specific RT–PCR to examine the expression of RASSF1A and RASSF1C in lung and breast tumor cell lines and in normal lung and breast epithelial cultures (Fig. 2). RASSF1A was expressed in normal lung epithelial cultures (NHBE and SAE cultures), in a normal breast epithelial culture (Fig. 2, C), but not in 32 (100%) of 32 SCLC lines, in 17 (65%) of 26 NSCLC cell lines, and in 15 (60%) of 25 (60%) breast cancer cell lines. Representative data are shown in Fig. 2. By contrast, RASSF1C was expressed in nearly all of the lung and breast cancer cell lines tested, with the exceptions of several lung and breast cancer lines with known homozygous deletions that include the RASSF1 locus. In resected lung adenocarcinomas, RASSF1A was expressed in only two of five cancers, while RASSF1C was expressed in all cancers (Fig. 2, C).

During RT–PCR analysis for RASSF1A, we frequently noted two closely spaced bands in RASSF1A-expressing tumors and in NHBE cultures (Fig. 2). We sequenced these RT–PCR products and found that the larger band corresponded to RASSF1A, while the smaller product represented a different transcript, RASSF1F (GenBank Accession #AF286217). This transcript skips exon 1C to produce an mRNA encoding a predicted truncated peptide of 92 amino acids ending within the DAG-binding domain (Fig. 1, D). The biologic function, if any, of RASSF1F is unknown. In nearly all of the samples, RASSF1F is expressed when RASSF1A is expressed. However, in some breast cancers and normal breast epithelial cultures (*see* Fig. 2 for examples), RASSF1A is expressed without RASSF1F expression.

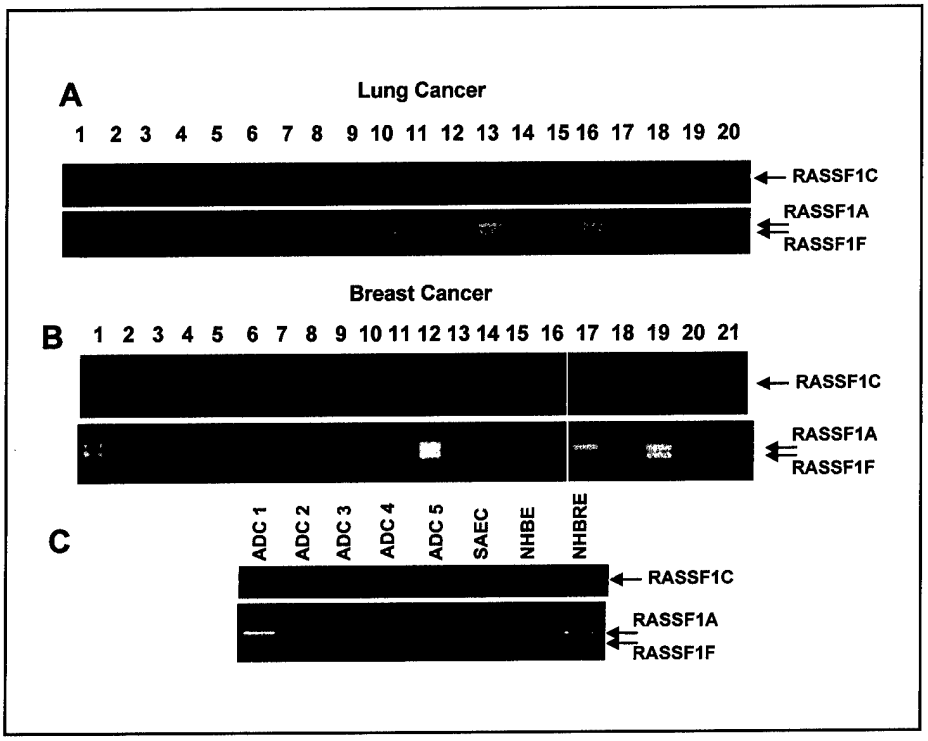

Fig. 2. RASSF1A and RASSF1C messenger RNA levels detected by isoform-specific reverse transcription–polymerase chain reaction (RT–PCR) in a sampling of lung cancer cell lines (**A**), breast cancer lines (**B**), and resected lung tumors and normal human lung and breast epithelial cultures (**C**). All RT–PCR products were separated on 2% agarose gels and were identified by staining with ethidium bromide. **Arrows** indicate location of transcripts. **A**) Lung cancer lines tested in **lanes: 1** = H157; **2** = H358; **3** = H727; **4** = H740; **5** = H748; **6** = H838; **7** = H1184; **8** = H1299; **9** = H1304; **10** = H1437; **11** = H1450; **12** = H1770; **13** = H1792; **14** = H1963; **15** = H1993; **16** = H2009; **17** = H2077; **18** = H2108; **19** = HHCC44; and **20** = HCC78. **B**) Breast cancer lines tested in **lanes: 1** = HCC38; **2** = HCC1187; **3** = HTB19; **4** = HTB20; **5** = HTB22; **6** = HTB23; **7** = HTB24; **8** = HTB25; **9** = HTB26; **10** = HTB27; **11** = HTB121; **12** = HTB129; **13** = HTB130; **14** = HTB131; **15** = HTB132; **16** = HTB133; **17** = HCC1395; **18** = HCC1428; **19** = HCC1569; **20** = HCC1806; and **21** = HCC2157. **C**) Resected lung adenocarcinoma samples (ADC 1–5) and cultures of normal small-airway epithelial cells (SAECs), normal human bronchial epithelial (NHBE) cultures, and normal human breast epithelial (NHBRE) cultures.

Methylation Status of the RASSF1A Promoter Region

Aberrant promoter methylation in tumors has been found to lead to the loss of gene expression of several tumor suppressor genes in human cancers *(14)*. To assess whether the loss of RASSF1A expression in lung cancer was the result of promoter hypermethylation, we determined the CpG methylation status in the 5′ region of RASSF1A (from –800 to +600 bp of the predicted RASSF1A transcript start site) by sequencing sodium bisulfite-modified DNA from eight lung cancer cell lines. All of the six lung cancer cell lines not expressing RASSF1A exhibited methylation of almost all CpG dinucleotide sites in the putative promoter region (data available online at the Journal website). The two lung cancer cell lines that did express RASSF1A either were not methylated at these CpG sites or showed limited methylation. By contrast, no methylation was found in CpG sites in the presumed RASSF1C promoter region of these eight cell lines.

To confirm that promoter hypermethylation contributes to the lack of expression of RASSF1A in the lung cancer cell lines, we assessed the effect of 5-aza-2′-deoxycytidine, a drug that inhibits DNA methylase, on RASSF1A expression. We exposed the RASSF1A-nonexpressing NSCLC line NCI-H157 to 5-aza-2′-deoxycytidine and found re-expression of RASSF1A by this cell line but little or no change in the expression of the housekeeping gene GAPDH or in the expression of RASSF1C (Fig. 3).

Methylation-Specific PCR Analysis of the Promoter Region of RASSF1A in Lung and Breast Cancers

To determine the methylation status of the promoter region of RASSF1A in primary lung and breast cancers, we used methylation-specific PCR analysis. Genomic DNA from a large number of primary resected NSCLCs, paired lung tissues resected from the same patients but not involved with the cancer, primary resected breast cancers, and a large panel of lung and breast cancer cell lines were treated with sodium bisulfite and tested for the presence of methylated and unmethylated CpG dinucleotides in the promoter region of RASSF1A (Fig. 4). All of the primary resected NSCLCs and non-tumor-paired samples contained unmethylated promoter sequences, which were expected because these resected tumors were not microdissected and were contaminated with stromal cells. However, 32 (30%) of 107 primary NSCLCs, 47 (100%) of 47 SCLC lines, and 19 (49%) of 39 primary breast cancers exhibited the methylated RASSF1A allele (Fig. 4; Table 1). By contrast, no methylated alleles were detected in 104 paired resected nonmalignant lung tissues (Fig. 4; Table 1).

We found a high frequency of methylated RASSF1A alleles in the panel of lung and breast cell cancer lines (Table 1). Because the lung and breast cancer cell lines represent essentially clonal populations of cancer cells without contaminating normal cells, we tabulated the frequency of the methylated and unmethylated RASSF1A alleles (Table 2). While the lung and breast cancer lines often derive from clinically more aggressive lesions than the average population of tumors *(19–21)*, our previous studies *(20,21)* have shown that cancer cell lines continue to retain the genetic alterations found in the uncultured cancer specimens from which they were derived. The presence of only the methylated allele is consistent with either the methylation of both parental alleles or the retention of the methylated allele and the loss of the unmethylated 3p allele. All of the SCLC cell lines

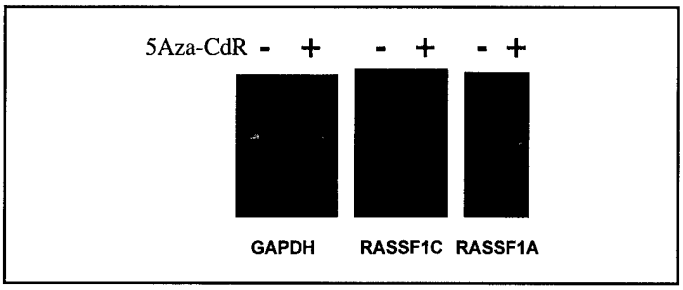

Fig. 3. Expression of RASSF1A after treatment of lung cancer cells with 5-aza-2′-deoxycytidine (5Aza-CdR). NCI-H157, a non-small-cell lung carcinoma (NSCLC) cell line that expresses RASSF1C but not RASSF1A, was grown in the presence (+ lanes) and absence (– lanes) of 0.5 μ*M* 5Aza-CdR for 48 hours. Total RNA was isolated, complementary DNA was prepared, and isoform-specific reverse transcription–polymerase chain reaction was performed for RASSF1A, RASSF1C, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control.

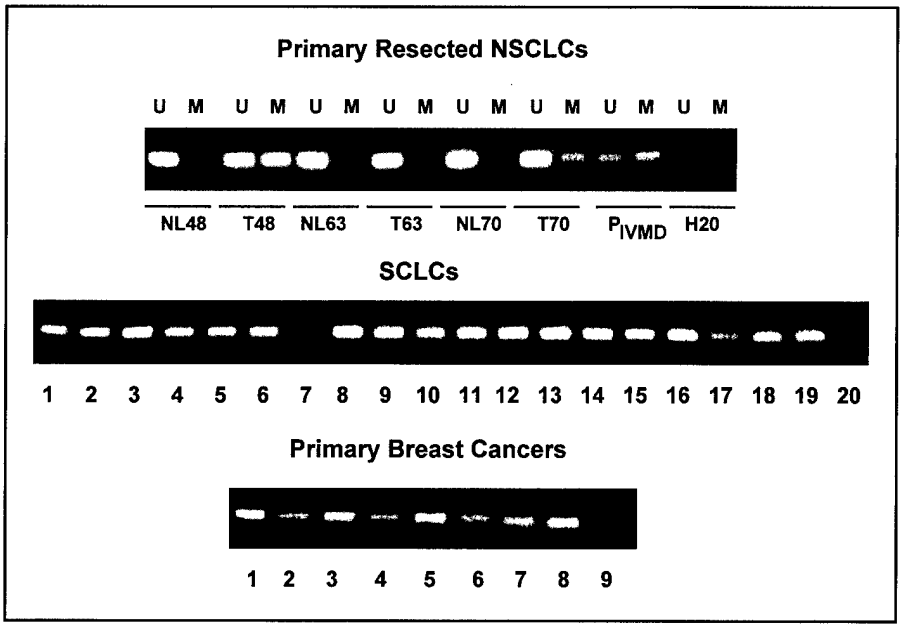

Fig. 4. Methylation-specific polymerase chain reaction (PCR) for the detection of methylated RASSF1A 5′ CpG sequences in primary resected non-small-cell lung carcinomas (NSCLCs) and their accompanying normal lung tissue (**upper panel**), small-cell lung carcinoma (SCLC) cell lines (**middle panel**), and primary breast cancers (**lower panel**). Representative samples are shown. For resected NSCLCs, U = results with primers specific for unmethylated sequences; M = results with primers specific for methylated sequences. NL = normal lung tissue; T = tumor; P = results with peripheral blood lymphocyte DNA, which is unmethylated or *in vitro* methylated (IVMD); and H20 = negative controls with water blanks. For SCLCs, **each lane** shows the PCR results for the methylated sequences from a different cell line. **Lane 20** is negative control. For the breast cancers, **each lane** shows the PCR results for methylated sequences from a different sample. PCR products were separated on 2% agarose gels, and bands were detected after staining with ethidium bromide.

Table 1. Frequency of methylation-specific polymerase chain reaction assay for detection of RASSF1A CpG island-methylated alleles in lung and breast cancers

DNA sample source*	No. tested	No. of methylation alleles (positive) (%)
Primary resected NSCLCs	107	32 (30%)
Corresponding nonmalignant lung	104	0 (0%)
NSCLC lines	27	17 (63%)
SCLC lines	47	47 (100%)
Primary resected breast cancers	39	19 (49%)
Breast cancer lines	22	14 (64%)

*NSCLC = non-small-cell lung carcinoma; SCLC = small-cell lung carcinoma.

Table 2. Presence of methylated and unmethylated RASSF1A alleles in 97 lung and breast cancer cell lines*

RASSF1A CpG genotype					
Methylated allele	Unmethylated allele	SCLC	NSCLC	BCCL	Total
+	+	0	4	4	8
+	–	47	13	10	70
–	+	0	10	7	17
–	–	1	0	1	2†
Total		48	27	22	97

*SCLC = small-cell lung cancer; NSCLC = non-small-cell lung cancer; BCCL = breast cancer cell lines.

†The two tumor cell lines with methylation-specific polymerase chain reaction genotypes lacking both methylated and unmethylated alleles (SCLC line NCI-H740 and breast cancer line HCC1500) were known to have homozygous deletions including the RASSF1 locus in chromosome region 3p21.3.

showed only the methylated allele or lacked RASSF1A entirely because of a homozygous deletion, consistent with the nearly universal 3p21.3 allele loss in SCLC *(1,20,33)*. Of the NSCLC cell lines, 13 (48%) of 27 (Table 2) had only the methylated RASSF1A allele, and 10 (37%) of 27 had only the unmethylated allele, consistent with a lower rate of 3p21.3 allele loss in this tumor type *(1)*. Likewise, 10 (45%) of 22 samples (Table 2) of breast cancer cell lines had only the methylated allele, and seven (32%) of 22 had only the unmethylated allele, again consistent with the rate of 3p21.3 allele loss found in breast cancer *(21)*. As expected, two tumor lines shown previously to have homozygous deletions involving the 3p21.3 region were negative for both the methylated and the unmethylated allele (Table 2) *(7,8)*.

For a subset of 61 lung and breast cancer cell lines, we performed both expression and methylation analysis and found a statistically significant association ($P<.001$, Fisher's exact test) between the presence of methylated RASSF1A alleles and the loss of RASSF1A expression. In 12 samples, RASSF1A was expressed in the absence of a methylated allele; in 44 samples, RASSF1A was not expressed in the presence of a methylated allele; in four samples, RASSF1A was not expressed in the absence of methylated allele (presumably because of some other inactivating mechanism); and in one sample (a breast cancer cell line), RASSF1A was expressed in the presence of both a methylated and an unmethylated allele. These data show the critical association of RASSF1A methylation with loss of RASSF1A expression.

We next assessed whether there was any association between RASSF1A promoter methylation and clinical findings in the patients with primary NSCLC. We found no statistically significant association between RASSF1A methylation and age, sex, tumor–node–metastasis (TNM) pathologic stage *(18)*, or tumor histology in 107 resected NSCLCs (data not shown). In addition, we found no statistically significant association between RASSF1A methylation and age, TNM pathologic stage, tumor histology, estrogen or progesterone receptor status, or HER2/Neu expression in 39 primary resected breast cancers (data not shown).

Survival among lung cancer patients differed by the methylation status of RASSF1A ($P = .046$) (Fig. 5). Also, by univariate analysis, in this group of 107 patients with NSCLC treated with an attempt at curative surgical resection, tumor (T1, T2, and T3), lymph node stage (N1 and N2), and reported weight loss were statistically significant predictors of adverse survival. Neither smoking history (yes/no or pack-years with 40 pack-year cutoff) nor treatment differences (all patients had surgical resection of lobectomy or pneumonectomy, and only five had prior radiotherapy or chemotherapy) accounted for the adverse survival. Because a multivariate analysis is of limited use with a small sample size, we performed a Cox proportional hazards regression analysis by use of RASSF1A methylation and the main univariate factors (tumor, lymph node stage, and weight loss). RASSF1A methylation was not found to be an independent prognostic factor of survival. However, this result could be due to small numbers because even lymph node stage (a known prognostic factor) was also no longer an independent factor in the analysis. Currently, we are studying a much larger cohort of NSCLC patients to determine whether RASSF1A methylation is an independent prognostic factor of survival.

Effect of Exogenous Expression of RASSF1A on Tumor Cell Phenotype

We examined the effect of RASSF1A on the tumor cell phenotype by three methods. We used anchorage-dependent colony

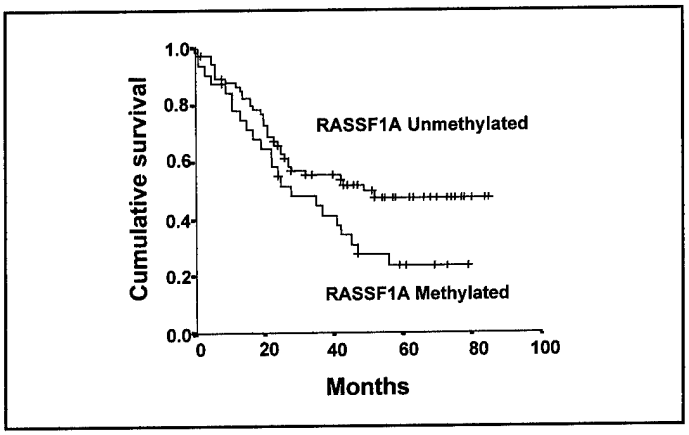

Fig. 5. Kaplan–Meier survival curve for 107 patients with resected non-small-cell lung carcinomas based on RASSF1A methylation status (32 methylated and 75 not methylated). For the patients with unmethylated RASSF1A alleles, the number of cases = 75, censored = 39, and events = 36, with a mean overall survival of 52 months (95% confidence interval [CI] = 44 to 59) and a median overall survival of 49 months (95% CI = 44 to 59); for the patients with methylated RASSF1A alleles, the number of cases = 32, censored = nine, and events = 23, with a mean overall survival of 37 months (95% CI = 27 to 46) and a median overall survival of 28 months (95% CI = 9 to 47). The log-rank test statistic for equality of survival distributions for RASSF1A methylation was 3.97, with *df* 1, $P = .0463$. The patients at risk for each group were: RASSF1A unmethylated—12 months (n = 63), 36 months (n = 34), and 60 months (n = 16); RASSF1A methylated—12 months (n = 24), 36 months (n = 13), and 60 months (n = 5).

formation as a measure of proliferation and anchorage-independent colony formation as a measure of malignant potential. We also directly assessed *in vivo* tumor formation.

We first cloned RASSF1A cDNA into pcDNA3.1+, an expression vector that contains a selectable marker, and transfected NCI-H1299 cells, which lack endogenous RASSF1A expression. After selection for 14–21 days, we determined colony formation of NCI-H1299 cells in both anchorage-dependent and anchorage-independent assays. Expression of RASSF1A in NCI-H1299 cells resulted in a 40%–60% decrease in anchorage-dependent colony formation and in an approximate 90% decrease in anchorage-independent colony formation compared with cells transfected with the pcDNA3.1 vector alone (Fig. 6, A). Because NCI-H1299 cells have an intragenic p53 homozygous deletion *(34),* transient expression of wild-type p53 can serve as a positive control for growth inhibition. Indeed, expression of wild-type p53 in NCI-H1299 cells resulted in a 80% and 95% reduction in colony formation in anchorage-dependent and anchorage-independent assays, respectively (Fig. 6, A). Several clones of NCI-H1299 cells transfected with RASSF1A were isolated in selective medium and were found to express RASSF1A by northern blot analysis (Fig. 6, B). Although the clones grew well *in vitro,* each had reduced anchorage-independent colony formation by approximately 90% compared with the vector-transfected control clones (Fig. 6, C).

To eliminate the possibility that the pcDNA3.1+ vector me-

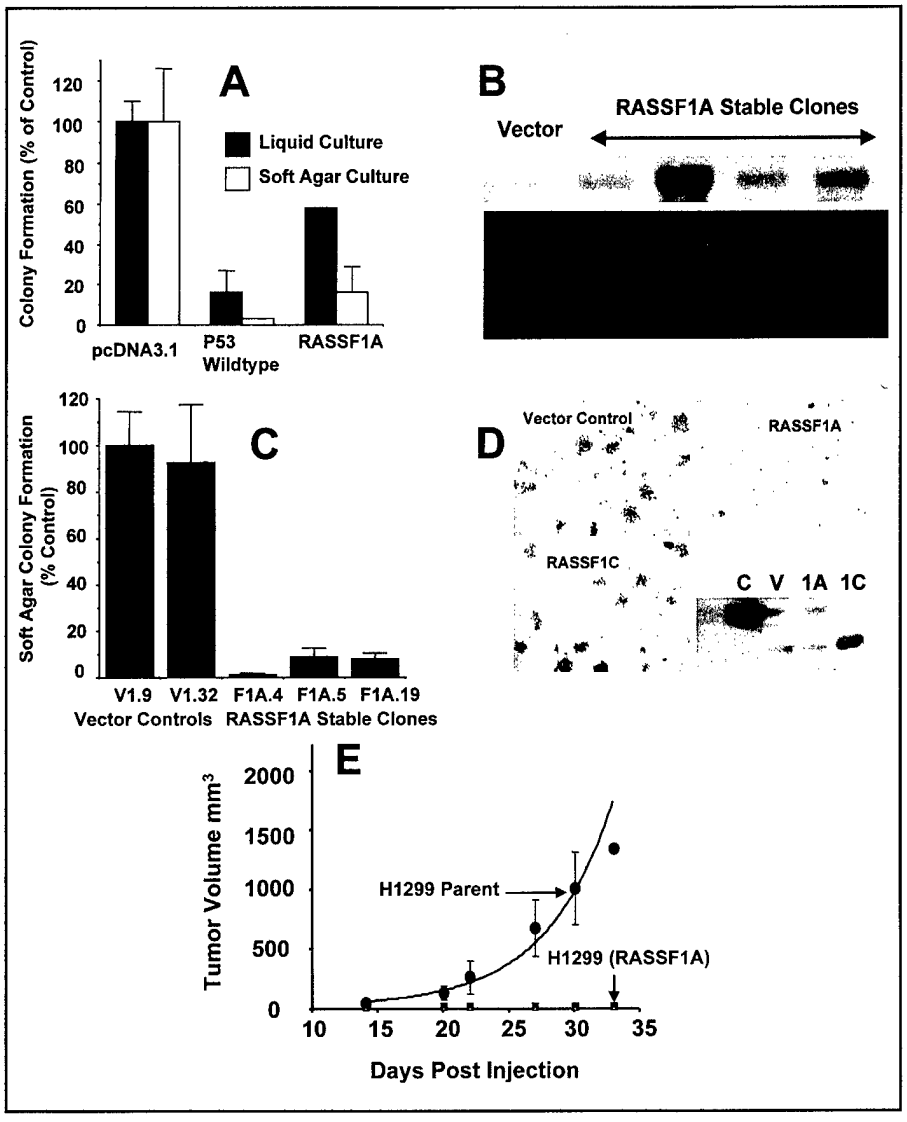

Fig. 6. Effect of RASSF1A on the *in vitro* and *in vivo* growth of the non-small-cell lung carcinoma (NSCLC) cell line NCI-H1299. **A**) Anchorage-dependent and anchorage-independent colony formation after transfection of NCI-H1299 cells with the empty vector (pcDNA3.1+) or pcDNA3.1+ expression vectors containing wild-type p53 or RASSF1A. For analysis of anchorage-dependent growth, after 2 days in nonselective growth medium, transfected NCI-H1299 cells were diluted into 100-mm^2 dishes with selective medium. Transfected cells were plated in liquid medium (for anchorage-dependent assays) or soft agar (for anchorage-independent assays) containing 800 μg/mL of G418. Colonies were stained with methylene blue in anchorage-dependent experiments after 14 days. Results represent the average of eight to 12 experiments in liquid medium and three soft-agar experiments. Standard deviations are shown or are less than 2%. **Solid bars** = anchorage-dependent growth (95% confidence interval [CI] = 0 to 36 for wt-p53 (wild-type) and 52 to 60 for RASSF1A); **open bars** = anchorage-independent growth (95% CI = 0 to 6 for wild-type (wt)-p53 and 0 to 39 for RASSF1A). **B**) Northern blot analysis of the RASSF1A expression in stable clones of NCI-H1299 cells transfected with the pcDNA3.1+ vector or pcDNA3.1+ containing RASSF1A complementary DNA (cDNA). The vector control (vector) and four separate clones with various RASSF1A messenger RNA levels are shown. Several of these clones were used in the anchorage-independent growth assay shown in **D.** Ethidium bromide staining of the ribosomal RNA is shown as a loading control. The clones were also verified to express the RASSF1A isoform by reverse transcription–polymerase chain reaction with the use of isoform-specific primers (data not shown). **C**) Soft-agar (anchorage-independent) colony formation in stable clones of NCI-H1299 cells transfected with the pcDNA3.1+ vector or pcDNA3.1+ containing RASSF1A cDNA. The means and standard deviations are shown. For each of the RASSF1A-expressing clones, the 95% CI = 0 to 4 for F1A.4, 2 to 16 for F1A.5, and 3 to 14 for F1A.19. **D**) NCI-H1299 cells were infected with the pBABEpuro retrovirus expression vectors containing either the vector control or the RASSF1A or RASSF1C cDNAs. Infected cells (10 000 per plate) were suspended in 0.33% agar, and the suspension was layered over a 0.5% agar base. Colonies greater than 0.2 mm in diameter were counted after 21 days. The **lower right panel** shows a representative western blot, developed with a rabbit antibody to the RASSF1-glutathione *S*-transferase fusion protein, to verify the expression of the RASSF1 proteins. C = positive control generated by transient transfection of NCI-H1299 cells with pcDNA3.1+ containing RASSF1A cDNA; V = infection of NCI-H1299 cells with the retroviral vector control (note runover from positive control); 1A = infection of NCI-H1299 cells with the retroviral vector containing RASSF1A; and 1C = infection of NCI-H1299 cells with the retroviral vector containing RASSF1C. **E**) Effect of RASSF1A on the *in vivo* growth of NCI-H1299 cells. Approximately 10^7 viable NCI-H1299 cells expressing RASSF1A were injected into the flanks of each of five previously irradiated BALB/c (nu/nu) nude mice. Tumor size was monitored over time, and size is shown in cubic millimeters. The average volume of tumors grown in more than 20 mice that were given an injection of vector-transfected NCI-H1299 cells is shown (H1299 parent). Mice that were given an injection of RASSF1A-infected NCI-H1299 cells grew no measurable tumors.

diated the growth-suppression effects, we infected NCI-H1299 cells with retroviral-expression vectors containing RASSF1A or RASSF1C and tested the ability of these cells to grow in an anchorage-independent manner. Cells expressing RASSF1A had a marked reduction in the ability to form soft-agar colonies compared with cells infected with the retroviral empty vector or the retroviral vector containing RASSF1C (Fig. 6, D). Cells expressing the retroviral vector formed 3200 colonies per 10 000 cells plated. RASSF1A-expressing cells formed only 19% of the vector control colonies, while RASSF1C formed 108% of the vector control. RASSF1A- and RASSF1C-infected cells grew well *in vitro* and showed no signs of toxicity or apoptosis (data not shown).

Finally, we tested the ability of the retrovirally infected NCI-H1299 cells to form tumors in nude mice. Cells transfected with the vector (parental cells) formed tumors rapidly (Fig. 6, E). By contrast, cells infected with RASSF1A retroviral vector and expressing the RASSF1A protein had much lower tumorigenicity *in vivo* (Fig. 6, E).

Discussion

We have found strong evidence that RASSF1A, but not RASSF1C, functions as a tumor suppressor gene that undergoes epigenetic inactivation in cancers by methylation of the CpG islands in the promoter region. Whereas normal lung and breast epithelial cells expressed both RASSF1A and RASSF1C, many lung and breast cancer cell lines did not express RASSF1A, although they did express RASSF1C. These tumor cell lines and uncultured primary lung and breast cancers frequently acquired RASSF1A 5′ CpG island hypermethylation, which was not found in paired lung tissues not involved with cancer from the same patient. Exposure of an NSCLC line to the methylase inhibitor 5-aza-2′-deoxycytidine restored expression of RASSF1A. We found that the loss of RASSF1A expression in a sample of resected NSCLCs was associated with decreased patient survival. Ectopic expression of RASSF1A by transfection by use of several different vectors into a cell line devoid of endogenous RASSF1A suppressed anchorage-independent growth (a measure of metastatic potential) and tumor formation in nude mice. Furthermore, although there was no evidence of *in vitro* morphologic changes, RASSF1A suppressed proliferation in an anchorage-dependent colony-formation assay. Independently, Dammann et al. *(15)* have recently reported similar results.

There is mounting evidence that tumor suppressor genes can be inactivated by tumor-acquired methylation of their promoter regions; indeed, this method of tumor suppressor gene inactivation may be more common than amino acid sequence-altering mutations *(14)*. We found only the methylated RASSF1A allele in 45%–100% of the tumor cell lines, depending on the tumor type, which was consistent with either methylation of both parental alleles or loss of the unmethylated allele. Because the 3p21.3 region, where RASSF1 is located, undergoes frequent allele loss in a variety of human tumors, including those of the head and neck, kidney, and cervix *(5)*, it will be important to extend the RASSF1A studies to these types of cancers. Although the methylation studies were prompted by the fact that we did not find any tumor-acquired, amino acid sequence-altering mutations in either RASSF1A or RASSF1C in our earlier study *(7)*, in this study, we did identify several polymorphisms in the RASSF1A-coding region of six NSCLC cell lines, several of which altered the amino acid sequence. Studies are in progress to determine whether any of these polymorphisms have functional consequences.

Analysis of the protein sequence of the RASSF1 isoforms revealed several domains that may aid in identification of specific cellular functions. The presence of a Ras-association domain in both RASSF1 isoforms suggests that these proteins may function as effectors of Ras signaling (or signaling of a Ras-like molecule) in normal cells. If so, the observation that RASSF1A can function as a tumor suppressor gene implies that RASSF1 acts in opposition to Ras-effector pathways that stimulate proliferation. Ras mutations rarely occur in SCLC or in breast cancer and are found in only approximately 30% of NSCLCs (usually in adenocarcinomas) *(2)*. Thus, the observation of RASSF1A methylation with the associated loss of expression in many tumors without Ras mutations suggests that inactivation of RASSF1A expression may be a tumorigenic mechanism that is distinct from the production of Ras mutations that lead to the activation of Ras signaling in tumors. However, it is important to note that, although many proteins have been identified that contain Ras-association domain motifs by database analysis *(9)*, the majority of these proteins have not been validated as *bona fide* Ras interactors. Therefore, studies are presently under way to assess the role of RASSF1 in Ras-dependent growth control.

Additional clues to the function of RASSF1A may be found in its protein structure. Kim et al. *(13)* found that both RASSF1A and RASSF1C possess a putative ATM kinase phosphorylation site in their common exon (Fig. 1), based on *in vitro* phosphorylation studies. Dammann et al. *(15)* isolated RASSF1 transcripts by use of a yeast two-hybrid assay, with the DNA repair protein xeroderma pigmentosum A as bait. These findings suggest that RASSF1 products may participate in the DNA damage response or in DNA damage-induced regulation of other cellular signaling events. The presence of a putative DAG-binding domain in RASSF1A but not in RASSF1C suggests studies to test the role of tumor promoters that interact with the RASSF1A isoform in a novel light: Tumor promoters act on proteins with DAG-binding domains by facilitating their movement to the cell membrane, thus allowing them to interact with the cell's signaling components. Thus, tumor promoters such as phorbol esters might be expected to move RASSF1A to the membrane, where it may act in its normal function as a growth suppressor until RASSF1A expression is lost.

Our previous work *(1,3,4,33)* has shown that 3p21.3 allele loss occurs early in lung cancer pathogenesis. Another study *(35)* has shown that promoter methylation of other tumor suppressor genes (e.g., for $p16^{Ink4A}$) can be detected in preneoplastic lung tissues or in histologically noninvolved lung tissue. RASSF1A promoter methylation may also represent a potentially important marker for the development of invasive lung and breast cancers. Because many smokers have genetic alterations in their respiratory epithelium as a result of damage by tobacco carcinogens *(3,4,33,36)*, the discovery of a marker such as RASSF1A promoter methylation may be of great use both for early detection and for prognosis in monitoring chemoprevention efforts. Furthermore, RASSF1A may represent another potential target for pharmacologic re-expression as a novel mode for cancer treatment.

Note added in proof: Five (71%) of seven primary resected uncultured SCLCs were positive for RASSF1A methylation by the methylation-specific PCR.

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The RASSF1A Tumor Suppressor Blocks Cell Cycle Progression and Inhibits Cyclin D1 Accumulation

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The RASSF1A locus at 3p21.3 is epigenetically inactivated at high frequency in a variety of solid tumors. Expression of RASSF1A is sufficient to revert the tumorigenicity of human cancer cell lines. We show here that RASSF1A can induce cell cycle arrest by engaging the Rb family cell cycle checkpoint. RASSF1A inhibits accumulation of native cyclin D1, and the RASSF1A-induced cell cycle arrest can be relieved by ectopic expression of cyclin D1 or of other downstream activators of the G_1/S-phase transition (cyclin A and E7). Regulation of cyclin D1 is responsive to native RASSF1A activity, because RNA interference-mediated down-regulation of endogenous RASSF1A expression in human epithelial cells results in abnormal accumulation of cyclin D1 protein. Inhibition of cyclin D1 by RASSF1A occurs posttranscriptionally and is likely at the level of translational control. Rare alleles of RASSF1A, isolated from tumor cell lines, encode proteins that fail to block cyclin D1 accumulation and cell cycle progression. These results strongly suggest that RASSF1A is an important human tumor suppressor protein acting at the level of G_1/S-phase cell cycle progression.

Loss of heterozygosity of chromosome region 3p21.3 is extremely common in lung, breast, ovarian, nasopharyngeal, and renal tumors (22, 27, 37, 39). Alterations at 3p21.3 are a very early event in primary cancer development, implying the presence of a tumor suppressor gene or genes in this location (20, 39). RASSF1 is one of eight predicted genes located in a minimal interval of 3p21.3 as defined by analysis of nested homozygous deletions found in tumor samples (24, 31). Two major splice forms of RASSF1, RASSF1A, and RASSF1C are expressed in normal human epithelial cells that derive from two different promoter regions (5, 6). The resulting mRNAs differ primarily in the selection of the first exon. RASSF1A contains an amino-terminal cysteine-rich region, which is similar to the diacyl glycerol binding domain (C1 domain) found in the protein kinase C family of proteins, and a carboxy-terminal putative Ras-association (RA) domain. RASSF1C is a smaller protein that lacks the amino-terminal C1 domain. Selective epigenetic inactivation of the RASSF1A promoter is an extremely common event in many human cancers. This includes 80 to 100% of SCLC cell lines and tumors (5, 6), 30 to 40% of NSCLC cell lines and tumors (5), 49 to 62% of breast cancers (5, 7), 67 to 70% of primary nasopharyngeal cancers (NPCs) (26), 91% of primary renal cell carcinomas (RCCs), and 100% of RCC lines (10). Ectopic expression of RASSF1A, but not RASSF1C, potently inhibits tumorigenicity of lung cancer cell lines, H1299 and A549 (5, 6), and an RCC line, KRC/Y (10). These results strongly suggest that RASSF1A may function as a tumor suppressor protein in many cells of epithelioid origin; however, the mechanism by which RASSF1A can negatively regulate tumor growth has not been determined.

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Normal epithelial cells require cell adhesion and the presence of appropriate growth factors to promote cell proliferation. These two signals act coordinately to regulate the G_1/S-phase cell cycle transition (4). G_1 includes a restriction point beyond which the cell is committed to undergo division, independent of extracellular growth regulatory signals. The retinoblastoma family of proteins (Rb, p107, and p130) are major gatekeepers of the G_1 restriction point. Hyperphosphorylation of Rb by cyclin D-CDK4 and cyclin E-CDK2 complexes is permissive for progression of proliferating cells into S phase. Alterations in expression or activity of components of this regulatory pathway are frequently associated with human tumors (32, 33). As an additional level of growth control, epithelial cells respond to loss of matrix adhesion by induction of programmed cell death (anoikis) even in the presence of growth factors that normally promote proliferation (16). Progression of epithelial cells to a tumorigenic state therefore requires bypass of regulatory checkpoints controlling both proliferation and survival.

Here, we begin to define the mechanism by which the RASSF1A tumor suppressor impacts growth regulation. Reintroduction of RASSF1A expression in lung and breast tumor-derived epithelial cells results in growth arrest but not apoptosis. This growth arrest correlates with inhibition of cyclin D1 protein accumulation, which likely prevents RASSF1A-expressing cells from passing through the Rb family cell cycle restriction point and entering S phase. Bypassing the requirement for cyclin D1 by artificially driving expression of cyclin A or expression of the viral Rb family inhibitor E7 relieved RASSF1A-induced cell cycle arrest. Regulation of cyclin D1 accumulation by RASSF1A is independent of the cyclin D1 promoter and likely occurs through inhibition of mRNA translation. Rare mutant alleles of RASSF1A, isolated from tumors and tumor-derived cell lines, were found to express proteins that cannot inhibit cyclin D1 accumulation or cell proliferation.

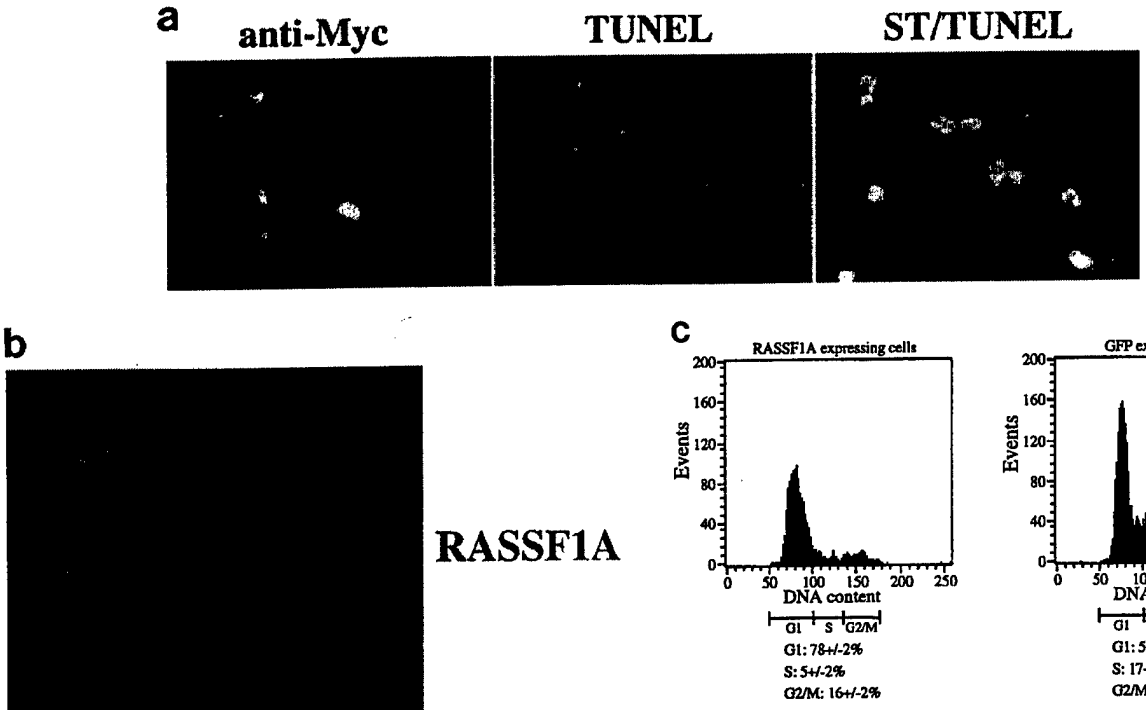


FIG. 1. RASSF1A blocks proliferation but does not induce apoptosis in human lung carcinoma cells. (a) H1299 cells were transiently transfected with Myc-tagged RASSF1A and placed in suspension cultures 24 h later. After 48 h of incubation in suspension cultures, cells were spun onto glass coverslips, fixed, stained with anti-Myc antibody to detect RASSF1A expression, and labeled by TUNEL to detect fragmented DNA. Cells were treated with 1 μM staurosporine for 4 h in suspension as a positive control for TUNEL (ST/TUNEL). (b) Twenty-four hours after transfection with the indicated constructs, H1299 cells were incubated for an additional 24 h in the presence of BrdU. RASSF1A expression was detected as in panel A. BrdU incorporation was detected with anti-BrdU antibody. An overlay image is shown. Quantitation by microscopic observation of three independent experiments is shown in Table 1. (c) Asynchronous H1299 cells were transfected with GFP or myc-RASSF1A. Forty-eight hours posttransfection, cells were trypsinized, fixed, and stained with propidium iodide. RASSF1A-transfected cultures were additionally stained with anti-Myc and fluorescein-conjugated anti-mouse secondary antibodies. Two-color FACS was used to determine the DNA content of GFP- or RASSF1A-expressing cells. Over 2,000 cells are scored for each analysis. The results shown are representative of three independent experiments. Quantitation of the population of cells in the indicated peaks is shown as a percentage of total events from the three experiments.

These mutations alter a putative mTOR/ATM family kinase substrate site and inhibit phosphorylation of RASSF1A, suggesting that RASSF1 activity is phosphorylation dependent. RNA interference (RNAi)-mediated inhibition of RASSF1A protein expression results in abnormal accumulation of native cyclin D1 protein in the absence of detectable changes in cyclin D1 mRNA levels. Together these results suggest that RASSF1A functions as a negative regulator of cell proliferation through inhibition of G_1/S-phase progression.

MATERIALS AND METHODS

Plasmids. pDCR-ras12V, −1745-CD1-Luc, pRC-cyclin D1, and pX-cyclin A have been described previously (1, 5, 8, 18, 19, 29, 38). Replication-defective retroviral particles derived from LXSN and LXSN-E7 were gifts from W. Wright (UT Southwestern Medical Center). pRK5myc-RASSF1A contains the full-length coding sequence of human RASSF1A as an *Eco*RI-*Xho*I fragment in pRK5myc2. pRK5myc-RASSF1C contains the full-length coding sequence of RASSF1C as an *Eco*RI-*Xba*I fragment in pRK5myc2. PRK5myc-RASSF1A(S131F), pRK5myc-RASSF1A(A133S), pRK5myc-RASSF1C(S61F), and pRK5myc-RASSF1A(A63S) were created by PCR-based site-directed mutagenesis and are otherwise identical to the wild-type versions. pGEX4T-1-Maxp1(229–455) contains an *Eco*RI-*Xho*I fragment of Maxp1 encoding residues 229 to 455 inserted in the *Eco*RI-*Sal*I sites of pGEX4T-1. PGEX4T-1-RASSF1A and pGEX4T-1-RASSF1C contain full-length coding sequences inserted as *Eco*RI-*Sal*I fragments into pGEX4T-1.

Cell culture and transfections. NCI-H1299 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. HME50-hTERT cells, a primary human diploid mammary epithelial cell line immortalized by hTERT expression (gift from J. Shay, UT Southwestern Medical Center), were grown in MCDB serum-free medium (Invitrogen) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech), 10 ng of epidermal growth factor (EGF) per ml, 5 μg of insulin per ml, 0.5 μg of hydrocortisone per ml, 5 μg of transferrin per ml, and 50 μg of gentamicin per ml (Sigma). HeLa cells were grown in DMEM supplemented with 10% calf serum. NCI-H1299 and

HME50-hTERT cells were transfected with Lipofectamine 2000 reagent (Invitrogen). HeLa cells were transfected with double-stranded RNA oligonucleotides by using Oligofectamine (Invitrogen).

Antibodies and immunofluorescence. Antibodies to RASSF1 were generated in New Zealand White rabbits inoculated with purified recombinant glutathione *S*-transferase (GST)-RASSF1(120–340) in RIBI adjuvant. Prior to use, RASSF1-specific antibodies were affinity purified from crude serum by standard methods. For bromodeoxyuridine (BrdU) incorporation experiments, 30 μM BrdU was added to transiently transfected cell cultures 24 h posttransfection. Following a 24-h incubation in BrdU, cells were fixed in 3.7% paraformaldehyde, permeabilized in acetone at −20°C for 5 min, and then treated with 2 M HCl for 10 min. BrdU incorporation was visualized with mouse monoclonal anti-BrdU and fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG). Myc-tagged RASSF1A expression was visualized with rabbit anti-Myc polyclonal antibodies (UBI) and rhodamine red X-conjugated anti-rabbit IgG (Jackson Laboratories), mouse 9E10 monoclonal anti-Myc antibody (Santa Cruz), and FITC-conjugated anti-mouse IgG (Jackson Laboratories), or chicken anti-Myc antibodies (Avery Laboratories) and cy5-conjugated anti-chicken IgG. Endogenous and ectopic cyclin D1 proteins were detected with rabbit polyclonal anti-cyclin D1 antibodies (UBI) and rhodamine red X-conjugated anti-rabbit IgG. Cyclin A expression was detected with rabbit anti-cyclin A antibodies (Santa Cruz) and Alexa Red-conjugated anti-rabbit IgG. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was carried out according to manufacturer's instructions (Roche). Biotinylated dUTP incorporation was detected with Texas red streptavidin (Jackson Laboratories). Fluorescence-activated cell sorting (FACS) of RASSF1A-expressing cells was performed by FACScan, with 10,000 cells collected for each assay, and analyzed by using CellQuest software (Becton Dickinson).

$^{32}P_i$ labeling. Forty-eight hours after transfection, cells were washed once and preincubated with phosphate-free modified Eagle's medium (MEM) for 10 min prior to addition of 0.5 mCi of $^{32}P_i$ to each 35-mm-diameter tissue culture plate. Following a 4-h incubation, cells were lysed (1% NP-40, 10 mM Tris [pH 7.5], 0.25 mM sodium deoxycholate, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 10% glycerol, 150 mM NaCl, 50 mM sodium fluoride, 1 mM orthovanadate, 80 mM β-glycerophosphate, and Roche protease inhibitor cocktail), and the RASSF1 variants were immunoprecipitated with anti-Myc antibody.

Gene expression assays. Luciferase activity assays were performed as previously described with the −1745-CD1-LUC reporter construct (19). Luciferase activity was normalized to β-galactosidase expression from cotransfected pCH110. For RNase protection assays (RPAs), total RNA was isolated from 10^6 cells with Trizol and further purified with a High Pure RNA isolation kit (Roche) according to the manufacturer's instructions. RPA was performed with the Riboquant RPA system (Pharmingen) together with the hCYC-1 multiprobe template set. A total of 5×10^5 cpm of the labeled probe set was hybridized with 2 μg of total RNA overnight at 56°C. Free probe and single-stranded RNA molecules were digested with RNase A. The hybridized probes were resolved on a 5% denaturing polyacrylamide gel and exposed to a PhosphorImager plate.

RNA interference assays. Double-stranded small interfering RNA (siRNA) oligonucleotides targeting RASSF1A were designed and prepared as described previously (12). The sequences used were 5′-GACCUCUGUGGCGACUUCA TT-3′ and 5′-UGAAGUCGCCACAGAGGUCTT-3′. Whole-cell lysates were taken for analysis 72 h following transfection.

RESULTS

RASSF1A induces G_1 cell cycle arrest. H1299 lung carcinoma cells, like many non-small-cell lung carcinomas, do not express RASSF1A due to loss of one allele and hypermethylation of the RASSF1A promoter on the remaining allele (5). These cells exhibit anchorage-independent growth (growth in soft agar) and can form tumors in athymic mice (5). We have previously shown that reintroduction of RASSF1A expression into H1299 cells is sufficient to inhibit the tumorigenicity of these cells (5), suggesting that loss of RASSF1A expression may be an obligate step for oncogenic transformation.

To begin to characterize the putative antioncogenic properties of RASSF1A, we first examined whether inhibition of tumorigenicity was through RASSF1A-induced apoptosis or cell cycle arrest. Transient transfection assays with native and epitope-tagged RASSF1A gave no indication of toxicity in adherent cells (5; data not shown). To determine if RASSF1A expression may be engaging an apoptotic program upon loss of anchorage, transiently transfected H1299 cells were assayed by TUNEL following incubation in suspension. H1299 cells were resistant to suspension-induced apoptosis up to the latest time point tested (72 h). As shown in Fig. 1, cells expressing RASSF1A gave no indication of an apoptotic response. In contrast, suspension cells treated with staurosporine showed strong labeling by TUNEL.

TABLE 1. Consequences of RASSF1 expression for BrdU incorporation and cyclin D1 protein expression

Protein	% of cells positive for expression[a]			
	H1299		HME50-hTERT	
	BrdU	Cyclin D1	BrdU	Cyclin D1
RASSF1A	20 ± 5 (10 ± 1)[b]	9 ± 1	13 ± 1	12 ± 1
RASSF1C	67 ± 8	44 ± 4	64 ± 1	58 ± 1
RASSF1A(S131F)	65 ± 11	62 ± 2	63 ± 2	59 ± 2
RASSF1A(A133S)	55 ± 5	49 ± 5	69 ± 4	68 ± 2

[a] Values are normalized to those of the vector control (set at 100%). Eighty to 90% of H1299 and HME50-hTERT vector-transfected cells were BrdU positive, and 70 to 90% were cyclin D1 positive. Unless otherwise indicated, all cultures were adherent. Means ± standard errors from at least three independent experiments are shown.

[b] The value in parentheses represents suspension.

To assess the consequences of RASSF1 expression on cell cycle progression, H1299 cells were assayed for BrdU incorporation following transient transfection with RASSF1A or RASSF1C. Expression of RASSF1A, but not RASSF1C, in adherent, subconfluent cells resulted in a dramatic inhibition of BrdU incorporation (Fig. 1b and Table 1). Similar results were observed in suspension cultures (Table 1). These observations suggest that ectopic expression of RASSF1A inhibits tumorigenicity through induction of cell cycle arrest.

To identify the nature of the cell cycle arrest, transiently transfected RASSF1A cells were examined for DNA content by FACS. RASSF1A-expressing cells were compared with green fluorescent protein (GFP)-expressing cells. Analysis of propidium iodide incorporation shows that, in contrast to GFP-expressing cells, the majority of H1299 cells expressing RASSF1A are in the G_1 phase of the cell cycle (Fig. 1c).

RASSF1A variants identified in tumor lines are uncoupled from G_1 arrest. Characterization of the RASSF1 locus in lung and breast carcinomas demonstrated that the RASSF1A isoform is not expressed in the majority of cell lines and tumors assayed due to methylation of the RASSF1A-specific promoter (5, 6). A mutation in the RASSF1 gene encoding a substitution of alanine to serine at position 133 of RASSF1A was detected in 12 breast and lung tumor samples that lacked methylation or had heterozygous methylation of the RASSF1A promoter region. The same mutation was detected in matched B-cell samples, suggesting it is a single nucleotide polymorphism rather than a somatic cell mutation. In addition, we unexpectedly cloned a cDNA from a human tumor cDNA library with a nearby mutation that encodes a substitution of serine131 to phenylalanine in RASSF1A. Serine 131 has been suggested to be a substrate for the ATM kinase (21), because the sequence

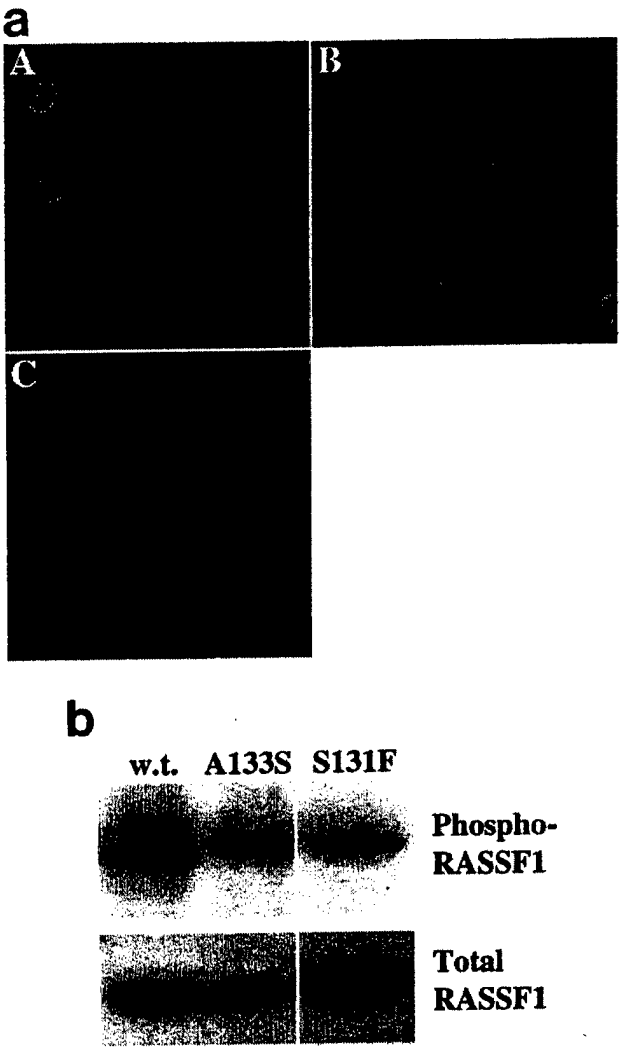


FIG. 2. RASSF1A variants isolated from tumor cell lines are unable to block proliferation. (a) H1299 cells were transfected with RASSF1A (A), RASSF1A(S131F) (B), or RASSF1A(A133S) (C) and processed as in Fig. 1b. Overlay images are shown with rhodamine red X-conjugated anti-rabbit IgG to detect the anti-Myc polyclonal antibody, and FITC-conjugated anti-mouse IgG to detect the anti-BrdU monoclonal antibody. Quantitation by microscopic observation of at least three independent experiments is shown in Table 1. (b) H1299 cells expressing the carboxy-terminal halves of the indicated RASSF1 variants were labeled with $^{32}P_i$ for 4 h. RASSF1 variants were immunoprecipitated, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and exposed to PhosphorImager plates to detect incorporation of $^{32}P_i$ (top panels). Total RASSF1 was detected by Western blotting with anti-Myc antibody (bottom panels). w.t., wild type.

WETPDLSQAEIEQK (amino acids 125 to 138 of RASSF1A) matches a putative ATM family phosphorylation site consensus sequence and a peptide with this sequence is an excellent ATM substrate in vitro (21). Both RASSF1A(A133S) and RASSF1A(S131F) were severely compromised in their ability to inhibit BrdU uptake in H1299 cells (Fig. 2a and Table 1). As shown in Fig. 2b, this defect correlates with the phosphorylation state of these proteins, which was significantly reduced compared to that of the wild type.

Bypass of Rb family proteins rescues cell cycle progression in RASSF1A-expressing cells. H1299 cells express Rb, but not p16. This suggests that Rb checkpoints may be difficult to engage in these cells. However, in addition to p16, the cyclin-dependent kinase inhibitors p21, p27, and/or p57 can contribute to negative regulation of CDK2 (33) and may help engage an Rb family checkpoint in H1299 cells. The E7 papillomavirus protein can bypass Rb family-dependent cell cycle regulation by directly inhibiting the interaction of Rb proteins with E2F transcription factors and other pocket-binding proteins (15). As shown in Fig. 3a, H1299 cells expressing E7 are resistant to RASSF1A-induced cell cycle arrest.

Cyclin A can directly activate CDK2 and participates in a feed forward amplification loop with E2F to drive cyclin E expression. Again, consistent with action of RASSF1A at the level of the Rb family restriction point, ectopic expression of cyclin A bypassed RASSF1A cell cycle arrest (Fig. 3a and b).

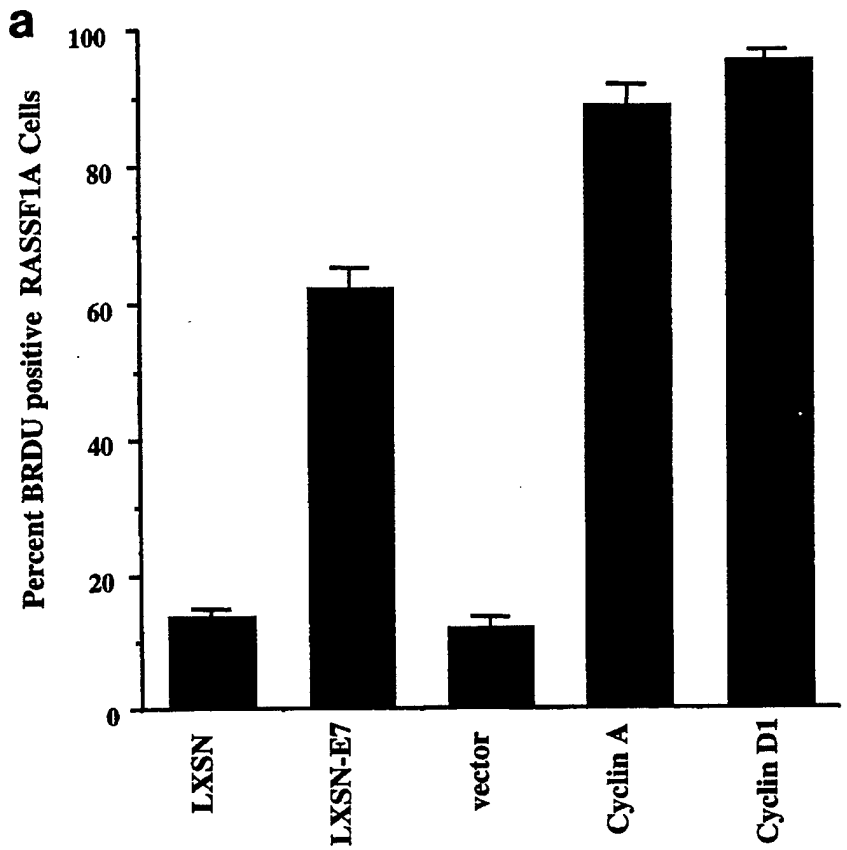
Oncogenic Ras does not bypass RASSF1A-induced growth arrest. RASSF1A and RASSF1C contain a carboxy-terminal region that may mediate direct binding to activated Ras family GTPases (5, 6, 36) and can interact weakly with Ras-GTP in vitro and when coexpressed with H-Ras12V in cells (36). To examine potential modulation of RASSF1A activity by Ras, we examined the consequence of H-ras12V expression on RASSF1A-induced growth arrest of human mammary epithelial cells immortalized by hTERT (HME50-hTERT) cells. As has been previously reported, in contrast to primary human fibroblasts, oncogenic Ras expression does not induce a "senescence-like" phenotype in HME-hTERT cells (13). As shown in Fig. 4, oncogenic Ras expression did not detectably alter RASSF1A growth-inhibitory activity in these cells.

RASSF1A inhibits accumulation of cyclin D1. Accumulation of cyclin D1 protein during G_1 contributes to bypass of Rb family restriction point control (32). Many mitogenic and oncogenic signal transduction cascades converge at the level of cyclin D1 accumulation, enhancing promoter activation (3, 18, 23), mRNA stability (11, 25), translation (28), and/or protein stability (1, 8). Consistent with a role in regulating G_1/S-phase progression, RASSF1A expression dramatically inhibits native cyclin D1 accumulation (Fig. 5a and Table 1). Expression of RASSF1C and RASSF1A variants had only modest effects.

The growth-inhibitory effects of RASSF1A are not limited to transformed cells, because inhibition of cyclin D1 accumulation and cell cycle progression by RASSF1A was also observed in HME-hTERT cells and primary mouse embryo fibroblasts (MEFs) (Table 1 and Fig. 4 and 5).

Regulation of the cyclin D1 promoter does not appear to be inhibited upon RASSF1A expression, because the steady-state

FIG. 3. Bypass of the Rb family cell cycle restriction point allows proliferation of RASSF1A-expressing cells. (a) H1299 cells expressing the indicated constructs together with RASSF1A were assayed for expression and BrdU incorporation. The percentage of cells expressing RASSF1A and incorporating BrdU was quantitated by microscopic observation. Bars represent the standard error from the mean of values obtained from three independent experiments. (b) H1299 cells were transiently transfected with RASSF1A together with cyclin A and assayed for expression of RASSF1A (A), cyclin A (B), and BrdU incorporation (C). Panel D is an overlay of images A to C.


a
Percent BRDU positive RASSF1A Cells
100
80
60
40
20
0
LXSN
LXSN-E7
vector
Cyclin A
Cyclin D1

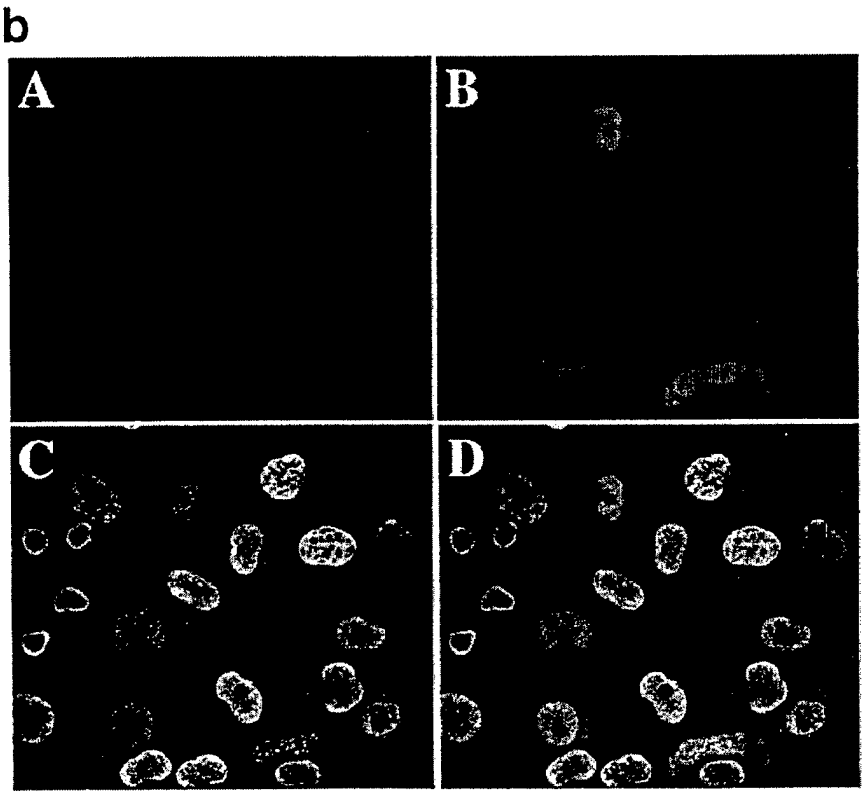

b
A
B
C
D

FIG. 3

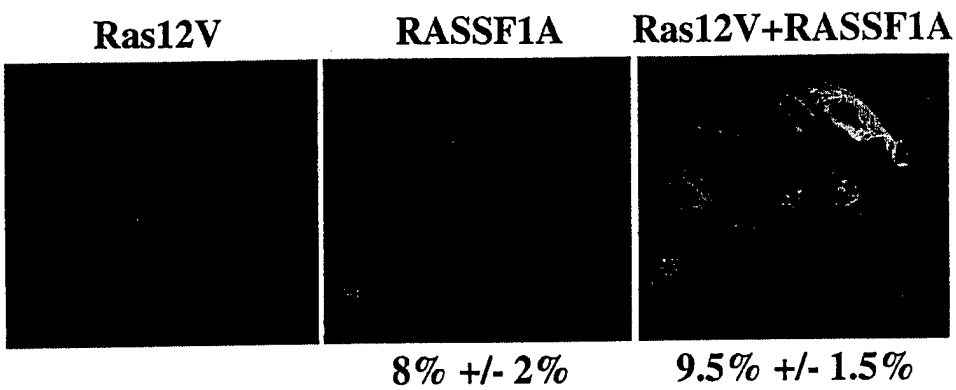


FIG. 4. Ras12V expression does not bypass RASSF1A-mediated growth arrest. HME50-hTERT cells were transfected with the indicated constructs and processed as described in the legend to Fig. 3b. The percentage of RASSF1A-expressing cells and RASSF1A+Ras12V-expressing cells incorporating BrdU is shown normalized to the BrdU incorporation frequency of cells expressing Ras12V alone (approximately 80% of total cells).

activity of a luciferase reporter driven by the cyclin D1 promoter (−1745 CD1-LUC) was not affected in H1299 cells. The activation of the cyclin D1 promoter by oncogenic Ras in HME-hTERT cells was also unaffected by RASSF1A (Fig. 5b). These observations suggest that RASSF1A may impact cyclin D1 protein accumulation posttranscriptionally. LLnL, lactacysteine, β-lactone, or MG132 proteosome inhibitors did not rescue cyclin D1 accumulation in RASSF1A-expressing cells, suggesting that the effects of RASSF1A are not achieved through alteration of cyclin D1 protein stability (data not shown). Together these results imply that RASSF1A can inhibit translation and/or stability of cyclin D1 mRNA, although this has not been directly demonstrated.

Ectopic expression of cyclin D1 from a viral promoter was sufficient to bypass RASSF1A-induced cell cycle arrest (Fig. 3a), suggesting that the inhibitory effects of RASSF1A on cell cycle progression are at the level of or upstream of cyclin D1 production. In addition, while RASSF1A can arrest the growth of wild-type MEFs, MEFs derived from cyclin D1 knockout mice (2) are insensitive to RASSF1A expression (Fig. 5c). Presumably, the cyclin $D1^{-/-}$ MEFS have bypassed the requirement for cyclin D1 expression through developmental compensation (14, 40). These results imply that the inhibitory effects of RASSF1A are mediated by cyclin D1-dependent checkpoints.

A dilemma facing interpretation of transient transfection analysis is the often unavoidable expression of proteins at much larger amounts in cells than would be produced from endogenous loci. To assess the contribution of native RASSF1A to regulation of cyclin D1 protein accumulation, we used siRNAs to inhibit expression of RASSF1A in HeLa CCL2 cells. These cells were chosen because they are highly amenable to treatment with siRNA, and they express detectable levels of endogenous wild-type RASSF1A. HeLa CCL2 cells are insensitive to RASSF1A-induced cell cycle arrest (Fig. 6a). This is likely due to the expression of papillomavirus E7 protein in these cells that bypasses Rb family cell cycle checkpoint control. This result is consistent with the observed bypass of RASSF1A-induced cell cycle arrest upon E7 expression in H1299 cells. Because Rb family function has been directly inhibited by E7, it is possible that upstream elements normally responsible for engaging the Rb checkpoint may still be intact in these cells. This has in fact been demonstrated empirically by the observation that an Rb-dependent checkpoint is engaged in HeLa cells upon elimination of E7 expression (17). Consistent with a role for endogenous RASSF1A in negative regulation of cyclin D1 accumulation, introduction of siRNA targeted to RASSF1A resulted in dramatically decreased RASSF1A expression and increased endogenous cyclin D1 protein accumulation compared to that in controls (Fig. 6b). Examination of the relative amounts of cyclin D1 mRNA in these samples also shows that inhibition of RASSF1A expression does not detectably alter the steady-state levels of cyclin D1 mRNA (Fig. 6c). This observation supports the hypothesis that native RASSF1A negatively regulates cyclin D1 accumulation through a posttranscriptional mechanism.

DISCUSSION

Loss of RASSF1A expression is a common event associated with many classes of human carcinomas. Reintroduction of

FIG. 5. RASSF1A prevents accumulation of cyclin D1 protein. (a) H1299 cells were transfected with RASSF1A or RASSF1C and stained with anti-cyclin D1 and anti-Myc antibodies. Overlays are shown with rhodamine red X-conjugated anti-rabbit IgG antibodies to detect rabbit anti-cyclin D and FITC-conjugated anti-mouse IgG to detect the 9E10 anti-Myc antibody. Quantitation by microscopic observation of at least three independent experiments is shown in Table1. (b) H1299 cells or HME cells were transfected with the indicated constructs together with a luciferase reporter construct driven by the human cyclin D1 promoter (−1745 CD1-Luc). Relative luciferase values are shown normalized to activities obtained with empty vector (H1299) or Ras12V alone (HME). Ras12V expression resulted in fourfold induction of luciferase activity in HME cells over baseline levels observed in serum-starved cells. Bars indicate the standard error from the mean of average values from three independent experiments performed in duplicate. (c) MEFs derived from wild-type and cyclin $D1^{-/-}$ mice were transiently transfected with the indicated constructs and assayed for BrdU incorporation as described in the legend to Fig. 1.

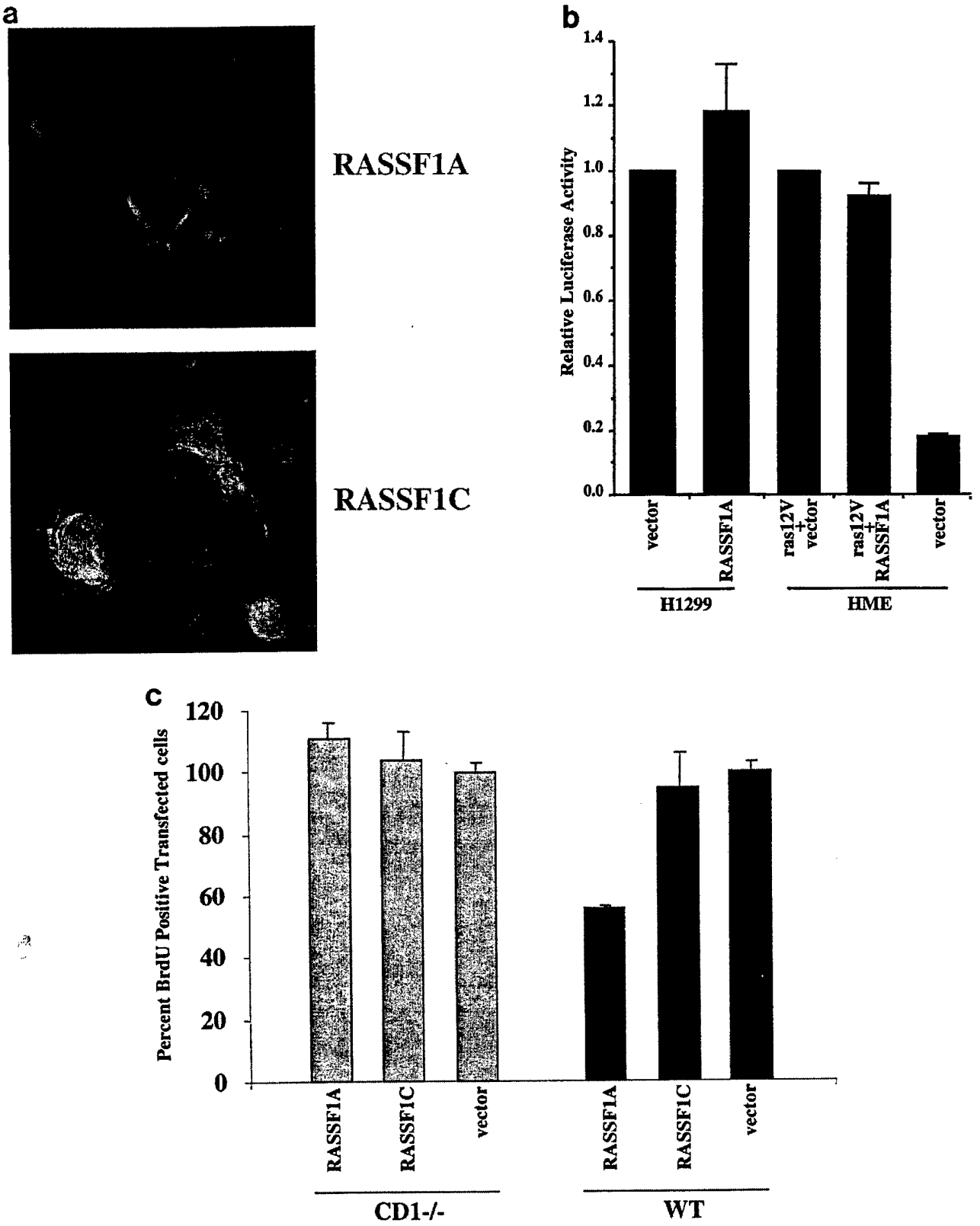

a
RASSF1A
RASSF1C
b
Relative Luciferase Activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
vector
RASSF1A
ras12V + vector
ras12V + RASSF1A
vector
H1299
HME
c
Percent BrdU Positive Transfected cells
120
100
80
60
40
20
0
RASSF1A
RASSF1C
vector
RASSF1A
RASSF1C
vector
CD1-/-
WT

FIG. 5

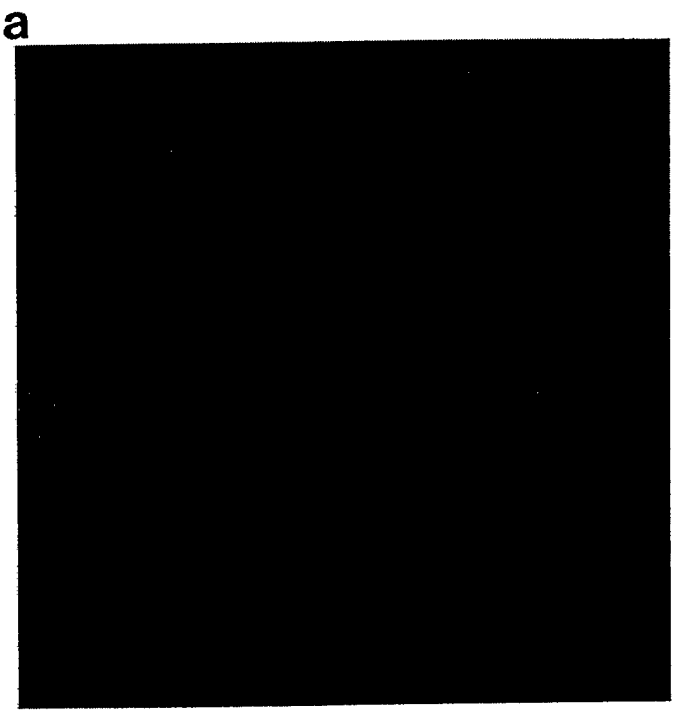


FIG. 6. Enhanced accumulation of cylin D1 in the absence of RASSF1A expression. (a) BrdU incorporation in RASSF1A-expressing HeLa cells was assayed as described in Fig. 1. (b) HeLa cells were exposed to siRNA specifically targeting the RASSF1A transcript for 72 h. siRNA directed against caveolin 1 was used as a negative control. Cyclin D1 levels in cells exposed to caveolin 1 siRNA are identical to those in untreated cells (data not shown). Whole-cell lysates were assayed for expression of the indicated proteins. Ten micrograms of total protein was loaded for each sample. (c) RPAs were performed to examine the relative amounts of cyclin D1 mRNA present in cells treated as described for panel b.

RASSF1A expression in tumor cells results in dramatic inhibition of tumorigenicity (5, 6). The results described here suggest that RASSF1A inhibits proliferation by negatively regulating cell cycle progression at the level of G_1/S-phase transition. RASSF1A-induced cell cycle arrest is accompanied by loss of cyclin D1 accumulation and can be relieved by ectopic expression of cyclin D1 cDNA. Significantly, inhibition of native RASSF1A results in abnormal accumulation of cyclin D1. This implies that RASSF1A modulates cell cycle progression through pathways regulating accumulation of cyclin D1 protein.

Accumulation of cyclin D1 is tightly regulated through multiple mechanisms, including promoter activation, mRNA stability, initiation of translation, and protein stability. The cyclin D1 promoter contains response elements for many mitogen-activated transcription factors, including AP1, NF-κB and Tcf/Lef (3, 18, 34). Regulation at the level of cyclin D1 mRNA accumulation can occur through destabilization elements in the cyclin D1 3′ untranslated region (UTR). AU-rich elements on the distal region of the cyclin D1 3′ UTR can be positively regulated by prostaglandin A_2 (25) and negatively regulated by phosphatidylinositol 3-kinase (11). In addition, initiation of translation of cyclin D1 mRNA is rapamycin sensitive in many cell types through an undefined pathway. Posttranslational control of cyclin D1 levels is mediated by phosphorylation-dependent polyubiquitination and degradation by the 26S proteosome (9). We have not yet identified the mechanism by which RASSF1A modulates cyclin D1 levels. However, we find that RASSF1A expression does not appear to affect cyclin D1 transcription or protein stability. This leaves the initiation of translation and/or mRNA stability as potential RASSF1A-sensitive steps in cyclin D1 regulation.

RASSF1C expression can be detected in most tumor cell lines (like H1299) that lack detectable RASSF1A expression (5). Further increasing the levels of RASSF1C protein does not greatly affect the proliferation of any cell type that we have tested. This includes several epithelium-derived tumor cell lines, normal mammary epithelial cells, NIH 3T3 fibroblasts, and 293 cells. In contrast to our observations, another group has reported that RASSF1C may induce apoptosis when overexpressed in 293T cells and NIH 3T3 cells (36). Although we did not observe this, we cannot rule out contrasting results due to unknown variables involving culture conditions or expression levels. We also found that RASSF1C did not significantly alter the levels of cyclin D1 protein accumulation. Our results suggest that the amino-terminal C1 domain of RASSF1A that is absent from RASSF1C is required for growth-inhibitory activity. The function of RASSF1C is unknown. It may be that RASSF1C can negatively modulate the activity of RASSF1A through unproductive association with cofactors; however, preliminary experiments suggest that RASSF1A expression is dominant to RASSF1C (L.S. and M.W., unpublished results).

RASSF1 is likely to be a member of a family of related proteins. RASSF1A is 60% homologous to mouse Nore1. The sequence of the human ortholog of NORE1, MGC10823, is present in GenBank, and KIAA0168 is an additional putative family member. It remains to be determined whether these family members play similar roles in the regulation of cell cycle progression; however, we have observed that expression of NORE1 has no detectable effect on the growth of adherent or suspension cultures of H1299 cells or HME-hTERT cells (data not shown). NORE1 can associate directly with Ras proteins in a stimulus-dependent manner (35). Like NORE1, all members of this family contain a carboxy-terminal putative RA domain (30). Although the RA domain of RASSF1A can weakly interact directly with Ras-GTP in vitro (36; our unpublished observations), it is unknown whether this interaction occurs in cells, and if so, what the consequences may be for RASSF1A activity. Given the tumor-promoting properties of activated Ras, it would be reasonable to speculate that Ras may negatively regulate RASSF1A activity to promote proliferation. However, we find that the effects of expression of RASSF1A are dominant to oncogenic Ras expression in human mammary epithelial cells. We were unable to detect any modulation of RASSF1A-induced growth arrest upon coexpresion with H-Ras12V. Although Ras is not likely a negative regulator of RASSF1A, our results leave open the possibility Ras may positively regulate endogenous RASSF1A activity.

From tumor cell lines expressing RASSF1A, we have identified rare germ line polymorphisms of RASSF1 with amino acid sequence alterations resulting in RASSF1A proteins [RASSF1A(S131F) and RASSF1A(A133S)] with decreased steady-state phosphorylation and decreased antiproliferative activity. These polymorphisms alter a putative phosphorylation site matching the consensus substrate site for ATM/ATR family serine/threonine kinases. It remains to be determined whether serine 131 is a bona fide phosphorylation site and whether the reduced activity of RASSF1A(S131F) and RASSF1A(A133S) is a direct consequence of reduced phosphorylation. The RASSF1A(A133S) variant is a consequence of a single-nucleotide polymorphism. Because this variant shows defective antiproliferative activity in our assays, it is possible that individuals carrying this polymorphism may have increased risk for development of some types of neoplastic disease. We have no way of knowing if the mutation we isolated encoding the S131F variant was a spontaneous somatic cell mutation occurring in a tumor or if it may be a rare polymorphism.

In summary, we have provided evidence indicating the mechanism of the growth-regulatory and tumor-suppressing activity of RASSF1A. Our results suggest that RASSF1A negatively regulates accumulation of endogenous cyclin D1 through a posttranscriptional mechanism leading to inhibition of cell cycle progression. Further studies need to be focused on understanding the regulation of cellular RASSF1A, as well as the molecular mechanism(s) by which RASSF1A impacts cyclin D1 accumulation and cell cycle progression.

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